Biofilms – Science and Technology

10.1007/978-94-011-1824-8

NATO ASI Series

Advanced Science Institutes Series

A Series presenting the results of activities sponsored by the NATO Science Committee, which aims at the dissemination of advanced scientific and technological knowledge, with a view to strengthening links between scientific communities.

The Series is published by an international board of publishers in conjunction with the NATO Scientific Affairs Division

A Life Sciences **B Physics**	Plenum Publishing Corporation London and New York
C Mathematical and Physical Sciences **D Behavioural and Social Sciences** **E Applied Sciences**	Kluwer Academic Publishers Dordrecht, Boston and London
F Computer and Systems Sciences **G Ecological Sciences** **H Cell Biology** **I Global Environmental Change**	Springer-Verlag Berlin, Heidelberg, New York, London, Paris and Tokyo

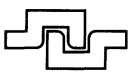
NATO-PCO-DATA BASE

The electronic index to the NATO ASI Series provides full bibliographical references (with keywords and/or abstracts) to more than 30000 contributions from international scientists published in all sections of the NATO ASI Series.
Access to the NATO-PCO-DATA BASE is possible in two ways:

– via online FILE 128 (NATO-PCO-DATA BASE) hosted by ESRIN, Via Galileo Galilei, I-00044 Frascati, Italy.

– via CD-ROM "NATO-PCO-DATA BASE" with user-friendly retrieval software in English, French and German (© WTV GmbH and DATAWARE Technologies Inc. 1989).

The CD-ROM can be ordered through any member of the Board of Publishers or through NATO-PCO, Overijse, Belgium.



Series E: Applied Sciences - Vol. 223

Biofilms – Science and Technology

edited by

L.F. Melo
University of Minho,
Braga, Portugal

T.R. Bott
University of Birmingham,
Birmingham, U.K.

M. Fletcher
University of Maryland,
Baltimore, Maryland, U.S.A.

and

B. Capdeville
Institut National des Sciences Appliquées,
Toulouse, France

Springer-Science+Business Media, B.V

Proceedings of the NATO Advanced Study Institute on
Biofilms – Science and Technology
Alvor, Portugal
18 May – 29 May, 1992

Library of Congress Cataloging-in-Publication Data

ISBN 978-94-010-4805-7 ISBN 978-94-011-1824-8 (eBook)
DOI 10.1007/978-94-011-1824-8

Printed on acid-free paper

CONTENTS

CHAPTER 6 - BIOFILMS IN FOOD PROCESSING

CHAPTER 7 - BIOFILMS IN WASTEWATER TREATMENT AND BIODEGRADATION OF WASTES

CHAPTER 8 - BIOFILMS IN WATER DISTRIBUTION AND INDUSTRIAL SYSTEMS

CHAPTER 9 - BIOFILM PREVENTION AND DESTRUCTION

FOREWORD

"Biofilms" or "microbial films" may be defined as communities of microorganisms adhering to surfaces, usually within a matrix of extracellular polymeric substances. They appear in many different practical situations in industry and medicine and can be either beneficial or detrimental.

Their use in bioreactors to degrade pollutant substances in liquid and gaseous effluents has expanded in the last two decades and is now one of the major waste water treatment processes. However, when biofilms build up in heat exchangers or cooling water towers, on reverse osmosis membranes or on ships' hulls, they create additional heat transfer, mass transfer and frictional resistances, increasing the capital and operating costs of such equipment. They may also cause contamination problems in the food processing industry.

In medicine, microbial films form on teeth, implants and prosthetic devices, sometimes with very serious, even fatal, consequences.

The purpose of this NATO Advanced Study Institute was to encourage discussion between scientists and engineers working on the different topics of basic and applied sciences related to the adhesion and development of biofilms on surfaces. Participants included both experienced researchers from academia and industry and younger graduate students and post-doctoral associates who are in early stages of biofilm research.

This book reflects the interaction between researchers from various fields, by including fundamental chapters on metabolism, microbial adhesion, process analysis and biofilm modelling, followed by the analysis of biofilm behaviour in wastewater treatment, food processing, cooling water systems and medicine. Chapters on prevention and destruction of biofilms, as well as on the laboratory and industrial techniques used to detect and study biofilm formation are also included. The final chapter contains short reports of the special discussion sessions that took place during the meeting.

The editors would like to express their gratitude to all the speakers and participants for their outstanding collaboration in the Advanced Study Institute as well as in the present book.

Special thanks are due to the support of the Scientific Affairs Division of NATO, which made the meeting possible.

The support of the following Portuguese institutions is gratefully acknowledged : University of Minho and its Biological Engineering Section, Junta Nacional de Investigação Científica e Tecnológica, Fundação Luso-Americana para o Desenvolvimento, Instituto Nacional de Investigacão Científica and Ministry of Defense.

The editors wish to thank Rosário Oliveira, Maria João Vieira and António Brito, from Minho University, for their invaluable help in the preparation of the Subject Index.

L. F. Melo T. R. Bott M. Fletcher B. Capdeville

Europe and USA, 1992.

Chapter 1

INTRODUCTION

INTRODUCTION TO THE PROBLEM OF BIOFOULING IN INDUSTRIAL EQUIPMENT

T.R. BOTT
School of Chemical Engineering
University of Birmingham
Edgbaston
Birmingham B15 2TT. UK

1. Introduction

Biofouling is a natural phenomenon and it is to be expected therefore that wherever suitable conditions exist biofilms may form. In industrial equipment the presence of biofilms may cause problems for the particular operation or process and the quality of the product may be adversely affected.

The areas of industrial activity, other than those discussed in subsequent papers, that may be affected by biofilm formation, are:

1. Cooling water systems
2. Biotechnology

In general the operating conditions associated with these technologies are conducive to biofilm formation. Nutrients are likely to be present, the temperatures encountered are those at which many micro-organisms thrive and for aerobic species air is readily available.

2. Cooling Water Systems

Many industries use water as a cooling medium including power generation, chemical manufacture, steel production and manufacturing processes generally. Three cooling water systems employ "fresh" water and include:

1. Closed recirculating
2. Open recirculating
3. Once through

2.1 CLOSED RECIRCULATING SYSTEMS

In these particular systems, as the name implies, the water recirculates but is contained in a sealed system so that it is not exposed to the atmosphere. Under these circumstances it is not possible for the water to become infected with bacteria or other micro-organisms. In this system biofilm formation is generally not a problem, unless leaks cause contamination.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 3–11.

2.2 OPEN RECIRCULATING SYSTEMS

In open recirculating systems the cooling water is usually "fresh" water obtained from a natural source, eg. a river or from a bore hole. Town's water is not often used on account of cost. After it has extracted heat from the process (usually in a heat exchanger) the water passes through some sort of evaporative cooler, eg. a cooling tower or spray pond. The evaporation of some of the water removes heat as latent heat in the vapour thereby reducing the temperature of the bulk. The water loss must be made up with fresh water. Figure 1 is a simplified sketch of a cooling water circuit employing a cooling tower.

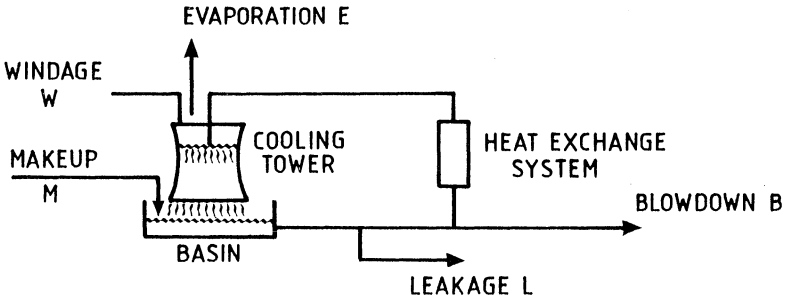


Figure 1. A simplified diagram of a cooling water system

As the cooling tower or spray pond is open to the atmosphere inoculation of the water with micro-organisms from the atmosphere together with possible nutrients and aeration can occur. The ecology of the system, if allowed to do so will reach an equilibrium and the circulating water may contain a spectrum of organisms including bacteria, algae and fungi. These micro-organisms are likely to thrive independently in different parts of the system relating to their preferred conditions. The bacteria may be most apparent on heat transfer surfaces, the algae in the basins or collecting areas at the base of the cooling tower or in the spray ponds. The fungi are most likely to be found on the packing inside the cooling towers that is there to produce effective mass transfer of water vapour to the air steam and hence to cool the bulk water.

In addition to evaporative losses, water is removed from the system due to leakage and there can be a loss from the top of the cooling tower in the form of droplets. As evaporation takes place the concentration of dissolved solids increases so that the solubility limit of the dissolved salts may be exceeded and precipitation can occur. Because the heat exchangers are the hottest part of the system inverse solubility salts can precipitate on the heat transfer surfaces causing additional problems. In order to reduce the overall dissolved solids concentration, water is bled from the system as "blowdown" to be replaced by fresh water (make up). The overall water balance (see Figure 1 for nomenclature) may be written as

$$M = W + E + L + B$$

Nutrients and micro-organisms will also enter with the make up water depending on the raw water source.

In order to overcome the potential problem of fouling on equipment surfaces and within pipe work, it is usual to employ a biocide to kill or reduce the level of biological activity.

2.3 ONCE THROUGH SYSTEMS

In once through systems water is abstracted from a source (say a river or lake), passed through the equipment to effect the cooling and then discharged again to the source. Provided the source (and sink) is sufficiently large the thermal effects are negligible. Similar problems to those encountered in open recirculation systems are likely to be experienced.

Once through systems usually employ "fresh" water but they may also operate with sea water. In the latter, quite apart from the effects of micro-organisms there is the strong possibility that larger creatures such as mussels, may inhabit the equipment surfaces.

2.4 PROBLEMS IN COOLING WATER SYSTEMS ASSOCIATED WITH BIOFILMS

The deposition of unwanted micro-organisms and the associated biofilms cause, amongst others, two major problems. They are:

1. Increased pressure drop (Δp) through the system. The presence of the biofilm represents a narrowing of the flow channel through which the water is being pumped. For a given volume flow reduced flow area represents an increase in velocity (v). Since $\Delta p \propto v^2$ even a modest amount of fouling will produce a relatively large pressure loss through the system.

Furthermore in comparison to the engineering surfaces of the equipment the biofilm may be considered "rough". The frictional resistance of rough surfaces is greater than smooth (or engineering surfaces). Additionally the visco-elastic character of biofilms means that energy is absorbed in the deforming process.

The overall result is an increase in pressure drop that under certain conditions could be substantial.

2. Reduced heat transfer efficiency. The presence of any layer of unwanted material on heat transfer surfaces represents a resistance to heat transfer, ie. the cooling efficiency of the system coolers is reduced.

In electric power production steam is passed through turbines to drive the generators. After passing through the turbine the steam is condensed in a condenser that is usually water cooled. The efficiency of the turbine is a function of the pressure drop across the turbine blades. For a given steam pressure the pressure fall across the turbine is a maximum when the pressure in the condenser is as low as possible. The efficiency of the power generation therefore depends on the steam pressure in the condenser that in turn depends on the efficiency of heat removal in the condenser. Foulants on the coolant side of the exchanger can adversely affect this process. Even small changes of pressure can represent significant changes in the cost of producing electricity.

In a particular 500 Mw power station the heat required to generate 1 kWh of electricity rose by 300 kJ for a very small change in condenser pressure.

Other industrial processes will be similarly affected to a lesser or greater degree. Quite apart from these two effects pressure drop and heat transfer resistance that might be substantial, other subsidiary problems may be manifest. Amongst these is the problem of corrosion.

As a biofilm develops the conditions under the biofilm may be very different from those in the bulk water away from the surface. There is a tendency for instance, for the pH to fall (indicating acid conditions), due to the metabolism of the micro-organisms. The result may be localised corrosion that can lead to pitting.

Micro-organisms and associated problems in cooling water systems are given in Table 1 (Kemmer 1988).

Higher life forms (macro-organisms) can cause problems such as the plugging of inlet screens.

2.5 REMEDIAL TECHNIQUES

It is possible to reduce the effects of biofilms in cooling water systems by cleaning (ie. the removal of biofilms from industrial equipment) or the prevention of their formation by the application of biocides.

2.5.1 *Cleaning of Surfaces.* Two opportunities for cleaning are available and include on line and off line cleaning. The former is accomplished while the equipment is still functioning, the latter requires the plant to be shut down and the heat exchangers say, opened for access.

On line cleaning can be achieved by physical cleaning or killing the organisms. Examples include:

1. The circulation of sponge rubber balls with the cooling water (the Taprogge system). In this technique sponge rubber balls pass through the heat exchanger and wipe the surface clean. The method is only suitable for tubular heat exchangers where the cooling water passes through tubes, eg. power station condensers. A case study (Bott 1990) demonstrates the effectiveness of the technology in a particular power station condensers. The "pay back time" for a retrofit system can be less than 12 months.

2. The use of shock doses of biocide that kill micro-organisms on the surface and cause sloughing of the biofilm. The concentration required varies from 10-200 mg/ℓ depending on the particular problem.

Off line cleaning is usually achieved by rodding and flushing techniques to dislodge the biofilm. The application of high pressure water jets may be necessary in some situations.

2.5.2 *Prevention.* The techniques described under Section 2.4.1 may be employed to prevent biofilms becoming established. However, it is likely that a continuous dosing of biocide will be necessary to maintain surfaces clear of biofilms. The dosing required will again be a function of the water condition.

Biofilms may become associated with or encouraged by, other forms of fouling, such as particulate deposition and crystallisation at the

TABLE 1. Typical micro-organisms and their associated problems (Kemmer 1988)

Type of organism	Type of problem
Bacteria	
1. Slime-forming bacteria	Form dense, sticky slime with subsequent fouling. Water flows can be impeded and promotion of other organism growth occurs.
2. Spore-forming bacteria	Become inert when their environment becomes hostile to them. However, growth recurs whenever the environment becomes suitable again. Difficult to control if complete kill is required. However, most processes are not affected by spore formers when the organism is in the spore form.
3. Iron-depositing bacteria	Cause the oxidation and subsequent deposition of insoluble iron from soluble iron.
4. Nitrifying bacteria	Generate nitric acid from ammonia contamination. Can cause severe corrosion.
5. Sulphate-reducing bacteria	Generate sulfides from sulfates and can cause serious localised corrosion.
6. Anaerobic corrosive bacteria	Create corrosive localised environments by secreting corrosive wastes. They are always found underneath other deposits in oxygen deficient locations.
Fungi	
Yeasts and moulds	Cause the degradation of wood in contact with the water system. Cause spots on paper products.
Algae	Grow in sunlit areas in dense fibrous mats. Can cause plugging of distribution holes on cooling tower decks or dense growths on reservoirs and evaporation ponds.
Protozoa	Grow in almost any water which is contaminated with bacteria; indicate poor disinfection.

surface. Techniques to combat these accumulations of unwanted deposits may also be required.

2.5.3 *The Cost of Biofouling in Cooling Water Systems.* A number of costs accrue from the presence of biofilms in industrial cooling water systems. They include:

1. Increased capital cost. In anticipation of a fouling problem it is usual to over design the heat exchangers (eg. condensers) in respect of clean conditions. The over design represents additional capital investment.

2. Additional operating costs. A number of additional costs resulting directly from the growth of biofilms can be identified.

2.1 Additional energy. Inefficient heat transfer usually means that extra energy is required. For instance the example of the power station condensers demonstrates that more energy is required to produce 1 kWh of electricity in the fouled condition compared to the clean condition.

The increased Δp discussed in Section 2.3 also means that increased pumping energy will be required. Doubling the velocity could mean a fourfold increase in pumping energy in addition to that produced by the increased roughness due to the biofilm.

2.2 Additional maintenance. The need to clean the heat exchangers represents additional costs. Furthermore the presence of biofilms can lead to other problems such as corrosion or leaking flanges or pump malfunction that will require maintenance that might not otherwise occur.

If the fouling problem is protracted and difficult to solve with existing equipment it may be necessary to install additional plant to overcome the problem (this cost might be considered as a capital charge or a maintenance cost).

2.3 The cost of additives. The need to apply biocides and possibly other chemicals represents a charge to operational costs.

2.4 Lost production. If heat exchangers have to be removed from service to allow cleaning and maintenance to be carried out this represents lost production, ie. a loss of revenue from the sale of the product. These losses may be even more unacceptable if an emergency shut down is involved.

2.5 Loss of employee morale. Continual problems with the operation of a heat exchanger can affect the morale of employees, particularly if there is a bonus scheme that is based on production. There can be attendant problems, such as a general lack of operating efficiency as a result.

2.6. Hidden costs. The use of biocides particularly, that persist in the environment, can lead to other costs when a treated water is discharged! These costs might involve surveillance and subsequent treatment if the raw water (ie. from a river) is required for drinking purposes.

3. Biotechnology

Biotechnology is the application of organisms, and their cellular subcellular or molecular components to the manufacture of commercial

products.

Biotechnological processes may be catalysed by living cells or specific compounds derived from them, such as enzymes. The micro-organisms, fungi or bacteria, may be grown in bulk for harvesting as biomass or employed to carry out specific chemical reactions. The products include pharmaceuticals, vaccines, diagnostics and a wide variety of valuable chemical compounds. Some aspects of food production can be classified as biotechnology, but the associated problems will be covered by Holah and Kearney (1992).

In general although not exclusively, biotechnological processes are carried out in what could be called stirred tank reactors. Fermentations are often carried out in such equipment when the reactors are usually called fermenters. A simple diagram of such equipment is given in Figure 2. The tank may vary from a few litres to hundreds of litres and is usually fitted with some form of stirrer or paddle to maintain uniform conditions. The tank wall may be fitted on the outside with a jacket or limpet coil so that heating or cooling may be accomplished. The design of the stirrer system will depend upon the conditions required in the bioreactor, ie. high turbulence or gentle mixing. The bioreactor may also be fitted with an internal coil for heating or cooling and baffles to increase turbulence.

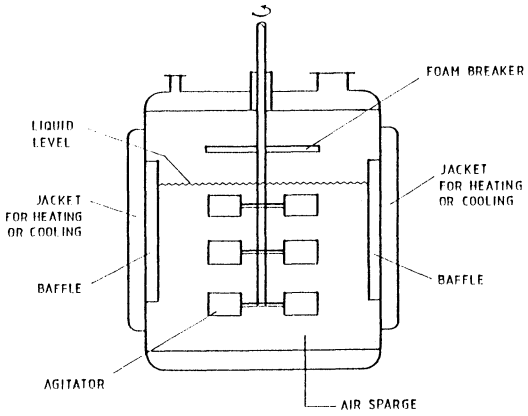


Figure 2. Diagram of a bioreactor (no internal coil shown for simplicity)

In the operation of a bioreactor the biological activity is to be encouraged so that the desired product is obtained and the yield made as high as possible. Good control of the process is required in order to maintain product quality. The favourable conditions lead inevitably to the formation of biofilms on the internal surfaces of the bioreactors including the vessel wall and the stirrer parts and baffles. The maximum growth of biofilm on these surfaces is often at the interface level (between the liquid broth and the air space). Substantial build up of deposits can occur on the stirrer shaft and the vessel walls at this point. The problem can be aggravated if foaming occurs that may be a result of the particular species being employed or the operating conditions.

Many bioreactions are carried out as a batch process with cleaning between batches but some may be operated on a continuous basis.

In many instances the presence of a biofilm is not a serious problem since it does not build up to excessive levels before the equipment is cleaned and sterilised for further operations. In others however, the presence of a biofilm can be a hindrance to effective operation, and a threat to product quality.

3.1 BIOFILMS ON THE STIRRER (AGITATOR)

The usual effect of biofilms on the surfaces of the agitator is to reduce the effectiveness of the mixing operation. Good mixing is essential to maintain effective control of the process.

3.2 BIOFILMS ON VESSEL WALLS AND EXTERNAL COILS

As with cooling water systems the presence of these deposits restricts heat transfer and as a result may affect batch times or even product quality. The presence of the biofilm on the surfaces may affect the kinetics of continuous growth. The consequence of which is a reduced return on plant investment.

3.3 DOWNSTREAM PROCESSING

During the discharge of the contents of a bioreactor pieces of the biofilm residing on surfaces may be dislodged and flow out with the discharge stream.

In the recovery of products downstream from the bioreactor these "lumps" of biofilm may represent a further nuisance, through the possible blocking of valves and filters.

3.4 REMEDIAL ACTION

Where high quality products are being produced as is often true in bioreactions, the use of additives may not be possible because of product contamination. It may not be even possible to use antifoaming agents to control the foam in the free space above the agitated liquid. The use of biocides as with cooling water is of course, not acceptable.

Close clearance agitators where the blades revolve close to the vessel wall, or in some instances scrape the surface, will serve to remove the biofilm as it forms. Such techniques can be employed to maintain trouble-free surfaces, but the retention of the high degree of engineering tolerances required may represent a maintenance problem.

The usual method of preventing biofouling on the surfaces is to manufacture to a high degree of finish. Smooth surfaces are generally less hospitable to micro-organisms than rougher surfaces that provide crevices in which colonies of micro-organisms can reside (relatively free of the effects of turbulence in the bulk liquid). Glass linings may be used to reduce the problem.

3.5 THE COST OF REMEDIAL ACTION

In general the improvement to the surface finish (or glass lining) can represent considerable additional capital cost compared to the plant fabricated to normal engineering standards. In addition high quality (high cost) maintenance will be required to retain the integrity of the surfaces.

Close tolerance agitation will also represent a higher capital investment and skilled maintenance will be essential if the full benefits of the installation are to be retained.

4. Concluding Remarks

The presence of biofilms in much industrial equipment represents an operating problem. Allowance for the problem in design results in increased capital investment and in some examples this can be substantial. In operation the biofilm is a nuisance that can lead to increased operating and maintenance costs and in some situations impair the quality of the product. The overall cost penalty is reduced competitiveness and lower profitability.

A better understanding of the science and technology associated with biofilms could lead to improvements in design of equipment with lower investment costs, and to reductions in operating, maintenance and associated costs.

5. References

Bott, T.R. (1990). *Fouling Notebook.* Instn. Chem. Engrs., Rugby.

Holah, J.T. and Kearney, L.R. (1992). *Introduction to biofilms in the food industry.* In Melo, L. *et al.* Biofilms - Science and Technology, NATO ASI Series, Kluwer Academic Publishers, Dordrecht.

Kemmer, F.N. (1988). *The Nalco Water Handbook.* 2nd edition. McGraw Hill Book Company, New York.

INTRODUCTION TO BIOFILMS IN WATER AND WASTEWATER TREATMENT

B. CAPDEVILLE
J.L. ROLS
National Institute of Applied Sciences
Department of Industrial Processes Engineering
Unité de Recherche Traitement Biologique
Complexe Scientifique de Rangueil
31077 TOULOUSE Cédex
France

Biological treatment consists of using micro-organisms to break down compounds which it is not desirable to discharge into the natural environment, by a cycle of transformations that can be opened on to the outside environment when the products obtained are considered to be non-polluting. In the case of the biological treatment of urban wastewater, the process requires a mixed population of micro-organisms to be placed in contact with the pollutant elements of the water to be treated, i.e. suspended solids and soluble substances of organic or mineral origin. In conventional biological wastewater treatment processes, this contact takes place in an aerated tank so as to encourage both the flocculation of the suspended solids with the micro-organisms, and the oxidation of soluble reducing substances. These so-called Activated Sludge procedures require prior treatment to remove sand and grease and allow for settling. Their operation relies above all on downstream clarification which clears the treated water and recycles the mixed liquors in the aerated tank (see figure 1).

In spite of all its advantages (gravity feed, high buffer capability), it is well known that this means of purification is limited by the close interdependence between the hydraulic residence time and the sludge age. If the liquid-solid separation conditions become degraded during running, the quantity of micro-organisms recycled at the head of the system is deficient and purification becomes insufficient. Moreover, the technologies can, in certain cases (particularly when there is only a limited land area available, and when odour and noise are a nuisance), pose problems when it is necessary to expand them or bring them into line with the new standards (notably concerning nitrogenous pollution for which the simultaneous increase in hydraulic residence time and sludge age means that the volume of the installations has to be multiplied by a factor of about three or four).

In the specific case of water for human consumption, such procedures are inadequate, considering the large volume of water to be made potable, the low pollutant concentrations and the risks of micro-organism leakage into the distribution network.

To get around the difficulties in maintaining activated sludge procedures and with a view to obtaining a more efficient liquid-solid separation for the effluent to be treated as a whole, procedures have been developed that use micro-organisms attached to a support, the aim being to dissociate sludge age from hydraulic residence time (Kinner et al., 1988).

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 13–20.

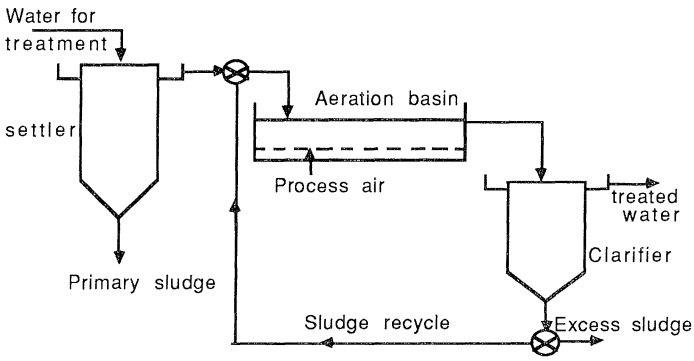


Figure 1. Basic cell of the activated sludge process.

We shall briefly mention the conventional fixed-culture procedures of the bacterial bed type (bacterial filter, biological disk, or equivalent procedures) invented at the beginning of this century, which have the advantage of rusticity but are of limited effectiveness, particularly because of their low specific surface area (about 100 m^2/m^3). A considerable technological advance was made, however, in the 1950's with the introduction of plastic support materials that were much more biocompatible than natural rock (wettability, porosity, density, etc.) and possessed a greater exchange surface area (of the order of 200 m^2/m^3). These new bacterial filters contain synthetic granular filling having a space coefficient sufficiently large to allow plates of excess biofilm to pass through and to leave room for a spontaneously occurring upflow of air that oxygenates the medium. The water to be treated is fed in at the upper part of the filter and travels to the inside by gravity. Since the 1980's, industrial installations have integrated bacterial filters into the secondary treatment to eliminate soluble organic matter (Norris et al., 1982 ; Orr et al., 1989) and into the tertiary treatment for nitrification (Parker et al., 1986). The same applications have also been described for biological disks (Boller et al., 1989).

New technologies appeared at the industrial stage around 1985 as the result of research and development aimed at associating the retention of colloidal and supracolloidal suspended substances with the oxidation of carbonaceous soluble pollution in the same apparatus. These biological filtration technologies, as they are called, make use of reactors in which the wastewater to be treated is forced through the filling instead of percolating freely under the effect of gravity as in bacterial filters. These biological filters contain a totally submerged, fine granular support occupying a height of 2 or 3 meters and having a specific surface area of 300 to 500 m^2/m^3, into which air is blown for ventilation.

In the first generation, the filtering media consisted of natural materials (such as clay, expanded schist, sand, carbon, etc.) with a density greater than that of the water to be treated, which made it indispensable to have a perforated base for the filling to rest on (see figure 2).

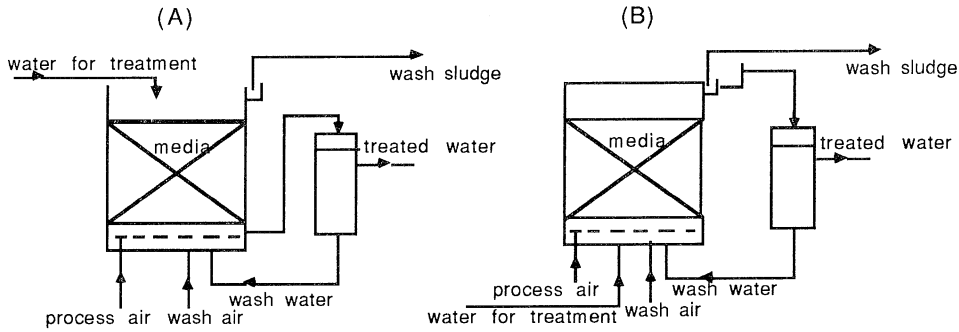


Figure 2. Flow diagram of biological filtration: (A) downflow, (B) upflow.

Among the basic procedures and their variants used in industrial operations, we would like to mention the following:

- the Biocarbone process from O.T.V. (France), with downflow of the wastewater,
- the Biofor process from Degrémont (France) with upflow of the wastewater,
- the Sulzer company's process (Switzerland), with downflow of the wastewater.

In addition to the advantages specific to these procedures (modularity, possibility of automation, small ground area occupied), it can be shown that the use of these fixed-culture reactors has other advantages in the treatment plant. In particular, clarification of secondary conventional treatment, and recycling of mixed liquor are no longer necessary. These properties result in far more compact stations which can be covered (insertion in urban environment, treatment of odours, minimum noise, etc.).

In spite of all the advantages of biological filtration over the Activated Sludge process, such plant have the major disadvantage of always working in transient mode because of the daily unclogging required. This leads to the use of systems having characteristics that optimize the restart phases: use of porous materials with well-implanted cultures, and automation of the washing cycles (usually performed in the night time).

In France, twenty or so installations for treating organic pollution operate with biofilters. They intervene in the treatment chain after a device for retaining suspended solids (see figure 3).

The treatment capacity of these stations is between 10 000 and 160 000 inhabitant equivalents (Gilles, 1989). To give an example, the following large French towns use this technology: Perpignan, Antibes, Monaco, Toulouse, Bordeaux, etc. A study carried out by CEMAGREF (Pujol, 1991) on behalf the French water agencies has shown that biofilters have the following operating characteristics on an industrial scale: hydraulic loading 2 to 5 $m^3/m^2.h$, volumic loading 3 to 7 kg $COD/m^3.d$, efficiency on COD 70% and sludge production 0.44 kg SS/kg COD eliminated. These processes have demonstrated their capacity to absorb daily fluctuations in flow and pollutant loading of the wastewater to be treated.

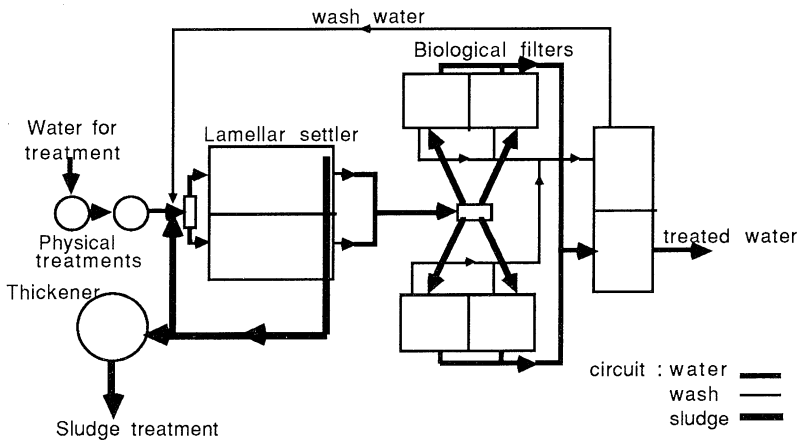


Figure 3. Flow diagram of wastewater treatment plant with biological fitration.

We have just seen the advantages of using submerged granular bed reactors for treating carbonaceous organic pollution. Another advantage is, of course, to be found in the field of nitrogenous pollution, given the new recommended standards. Nitrifying bacteria can attach to a granular medium and, although their growth rate is low, it is possible to use them in a biological filter to eliminate ammonia (Rogalla et al., 1988). Thus, the initial expansion to bring water treatment stations into conformity has consisted of adding a tertiary, wastewater nitrification process using fixed cultures. Denitrification can also be performed in a biological filter (Polprasert et al., 1986), allowing total elimination of nitrogen from wastewater at secondary treatment level in a station. The conventional system (see figure 4) advocated is recycling of the nitrified effluent in an anoxic filter (Jimenez et al., 1987) similar to single-sludge nitrification and denitrification. The downflow anoxic biological filter performs denitrification (reduction of nitrates to molecular nitrogen and oxidation of soluble organic substances) and retention of suspended solids in the water to be treated. The biological filter with upward ventilation then ammonifies and nitrifies the wastewater. There have recently been a few installations set up on an industrial scale (Gilles et al., 1987 ; Mange et al., 1989).

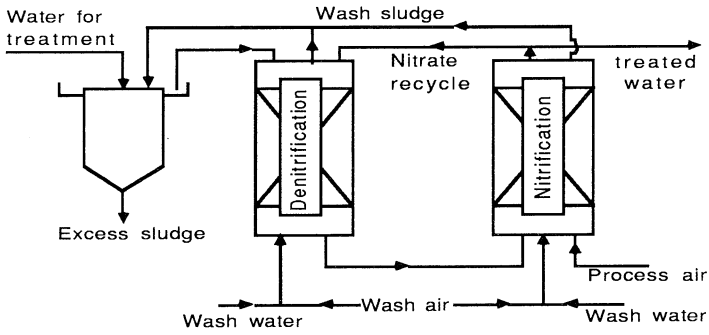


Figure 4. Flow diagram of nitrification and denitrification process using biological filters.

But the first use of fixed cultures for denitrification on an industrial scale was in the field of water to be made potable. Biological procedures were added to the existing treatment systems in order to resorb nitrates from groundwater which, in certain intensive farming regions, exceeded the levels acceptable for drinking water. These procedures, derived from biofiltration processes for urban wastewater, are specific to the treatment of potable water (in particular, the material is of quality suitable for contact with food). These procedures are: Nitrazur from the Degrémont company (Richard et al., 1986) and Biodénit from the O.T.V. Company (Ravarini et al., 1988). They only concern the biological reaction, filtration not being concerned.

We have just seen the interest of fixed culture processes using a fixed granular support in the spheres of urban wastewater and potable water treatment. But, since 1986, these processes have also been in use in Europe for the anaerobic treatment of industrial waste, e.g. distillery and starch wastewater. The processes use the plastic materials originally developed for bacterial filters and allow the treatment of water with concentrations up to 40 g COD/l with an efficiency of 95% (Seyfried et al., 1989 ; Racault, 1989).

The second generation of submerged filters, currently in the pre-industrialization stage, involve the development of technologies implementing floating supports composed of synthetic materials having a density lower than that of water (e.g. polystyrene). These technologies overcome some of the disadvantages of the previous systems, notably in the filter regeneration phase for which large amounts of fluid (water and air) and power can be consumed to loosen the bed and wash away excess sludge. The procedure that takes advantage of this technological advance is the Biostyr from the O.T.V. company, which operates with upflow of both the water to be treated and the process air through a floating support kept inside the reactor by a perforated lid. The possibilities of this procedure in the intensive elimination of pollution containing nitrogen (nitrification-denitrification) and phosphorus, investigated on a pilot scale by Goncalvez et al. (1992), lead us to predict its interest for compact stations treating pollution containing carbon, nitrogen and phosphorus.

With a view to improving fixed culture procedures, studies were undertaken on the possibilities of fluidizing certain types of fine natural materials (sand, pumice stone, clay, etc.) so as a) to increase the exchange surfaces (from 500 m^2/m^3 for the fixed bed to 1500 m^2/m^3 for the fluidized bed) and thus the quantity of micro-organisms fixed per unit volume and b) to have system with a continuous, non-clogging supply. Although the fluidization is performed by the upflow of the water to be treated assisted by recycling (upflow velocity 10 m/h), the procedure may comprise two (material - liquid) or three (material - liquid - gas) phases. The fluidized bed technologies have reached an industrial scale for the case of two-phase systems (anaerobic process), but the number of installations remains very limited. For example, MacDonald (1989) reports the implementation of a fluidized sand bed reactor for the tertiary denitrification of urban wastewater in a plant treating 25 000 m^3/d. For the treatment of industrial wastewater, the Degrémont company offers the Anaflux methanization procedure. An industrial plant is known to exist in Spain for treating brewery waste (Oliva et al., 1989).

As far as three-phase fluidized beds are concerned, oxygenation systems have been developed such as the Oxitron system (Dorr-Oliver Ltd., Sutton et al., 1981) and the U-tube (Jeris et al., 1981). However, the development of these procedures has remained at the pilot stage except for a few industrial realizations (Nutt et al., 1984 ; Hare et al., 1988). The reasons for this are the following : a) difficulty in distributing gaseous and liquid fluids loaded with suspended solids

over large base areas, b) great instability of the fluidized bed due to the accumulation of biofilm on the materials and c) abrasion and loss of materials.

These factors can be summed up in the inability of natural materials (whose characteristics are high density and low grain size) to maintain their volume properties as time passes, and the interference of the fluidized material in the gas-liquid transport (Diniz Leao, 1984 ; Hatzifotiadou, 1989). It is nevertheless possible to avoid these difficulties by using materials suited to this type of reactor, particularly composite thermoplastic materials such as those developed in our institute. These granular materials, called OSBG (Optimized Support for Biological Growth), have a diameter of a few millimeters and a density close to that of water (about 1100 kg/m^3). It is thus possible to keep a three-phase fluidized reactor in stable conditions (without loss of materials), developing a thin, very active biofilm, the interest of which will be explained in the over article published in this volume (Capdeville et al., 1992). To give an example, this process used for the treatment of organic pollution in urban wastewater gave an elimination efficiency of 85% for an applied loading of 15 kg COD/m^3.d (Lertpocasombut, 1991). This pilot system has also been used for the denitrification of drinking water (Lazarova et al., 1992).

The knowledge acquired through the research on biofilm reactors enables new systems to be envisaged that are more compatible with urban wastewater systems (where considerable amounts of suspended solids are present). The approach consists of keeping both the advantages of cultures attached to a granular medium and those connected with free cultures. In this case it is possible to develop mixed cultures in reactors of conventional form, i.e. with gravitational liquid feed, but into which fixed submerged support material is introduced. The interest of this procedure lies in the prospect of simultaneous elimination of pollutants containing carbon and nitrogen (nitrification) in the same tank (Hegemann, 1984 ; Schlegel, 1988). The presence of support material enables the mass loading to be reduced and the sludge age to be increased independently of the hydraulic residence time. For the moment, a few full-scale trials have been carried out with promising results (Bonhomme et al., 1989). A variant of this procedure is the use of granular material kept moving by the mixing caused by the ventilation of the tank. These so-called turbulent bed reactors have been studied at laboratory level (Nicol et al., 1988).

In conclusion, water treatment and purification procedures have fundamentally evolved in the last 15 years towards the use of fixed cultures. These procedures have the advantage of treating a large flow in the same reactor volume, thus allowing stations to be more compact (notably for the simultaneous elimination of carbon, nitrogen and phosphorus) whilst dealing with the problems of odour reduction, noise abatement, the integration of the installations in the landscape, and the modularity and automation of the plant. The first generation of such procedures consisted above all of industrializing fixed submerged granular beds of the sinking or floating type. At present, new perspectives have been opened up by improvements made to these techniques or by the use of materials with the same characteristics but mobile in the liquid phase to be treated.

In all these processes, a fundamental question as yet unanswered concerns the reactivity of a biofilm itself and whether high concentrations of micro-organisms need to be maintained or not. In the over article presented in this volume (Capdeville et al. 1992), we shall discuss further the interest of thin biofilms and the limits of the conventional diffusion theory in explaining the biological processes taking place in a biofilm.

References

Boller, M., Gujer, W. and Nyhuis, G. (1990) "Tertiary rotating biological contactors for nitrification" Wat. Sci. Tech., 22,1/2, 89-100.

Bonhomme, M., Rogalla, F., Boisseau, G. and Sibony, J. (1990) "Enhancing nitrogen removal in activated sludge with fixed biomass", Wat. Sci. Tech., 22,1/2, 127-136.

Capdeville, B., Nguyen, K.M. and Rols, J.L. (1992) "Biofilm modelling : structural, reactional and diffusional aspects", in "Biofilms : Sciences and Technology", T. R. Bott, B. Capdeville, M. Fletcher and L. Melo (Eds), Kluwer Academic Publishers.

Diniz Leao, M.M. (1984) "Contribution à l'étude du comportement des particules dans un réacteur biologique de dépollution à lit fluide", Ph. D. Thesis n.134, INSA Toulouse, France.

Gilles, P. (1990) "Industrial scale applications of fixed biomass on the mediterranean seaboard design, operating results", Wat. Sci. Tech., 22,1/2, 281-292.

Gilles, P. and Bourdon, Y. (1987) "Nitrification and denitrification with fixed bacteria", L'Eau, l'Industrie, les Nuisances, 93.

Goncalvez, R.F. and Rogalla, F. (1992) "Biological phosphorus removal in fixed film reactors", Conference on "Gérer les eaux usées en zones littorales", Montpellier, France, Mars.

Hare, R.W., Sutton, P.M., Mishra, P.N. and Potochnik, K.F. (1988) "Utilization of fluidized bed biological treatment at General Motors facilities : pilot and full scale results", Proceeding of the Industrial Waste Symposia, Water Pollution Control Federation, Dallas, USA.

Hatzifotiadou, O. (1989) "Contribution à l'étude de l'hydrodynamique et du transfert de matière gaz-liquide dans un réacteur à lit fluidisé triphasique", Ph. D. Thesis n.83, INSA Toulouse, France.

Hegemann, W. (1984) "A combination of the activated sludge process with fixed film biomass to increase the capacity of waste treatment plants", Wat. Sci. Tech., 16, 119-130.

Jeris, J.S., Owens, R.W. and Flood, F. (1981) "Secondary treatment of municipal wastewater with fluidized bed technology", in "Biological fluidized bed treatment of water and wastewater", P.F. Cooper and F. Atkinson Eds., Ellis Horwood, Chichester, England, pp. 112-120.

Jimenez, B., Capdeville, B., Roques, H. and Faup, G.M. (1987) "Design considerations for nitrification and denitrification process using two fixed bed reactors in series", Wat. Sci. Tech., 19, 139-150.

Kinner N.E. and Eighmy, T.T. (1988) "Biological fixed-film systems", J. Wat. Poll. Control Fed., 60, 6, 824-828.

Lazarova, V., Capdeville, B. and Nikolov, L. (1992) "Influence of seeding conditions on nitrite accumulation in a fluidized bed biofilm reactor for drinking water denitrification", IAWPRC 16th Biennial International Conference, Washington, USA, May.

Lertpocasombut K. (1991) "Epuration carbonée par film biologique mince dans un réacteur à lit fluidisé triphasique", Ph.D. Thesis n.159, INSA Toulouse, France.

MacDonald, D.V. (1990) "Denitrification by fluidized biofilm reactor", Wat. Sci. Tech., 22,1/2, 451-462.

Mange, P. and Gros, H. (1990) "Traitement biologique d'eaux usées communales par biofiltration sur matériau granuleux et nitrification sur support immergé", Wat. Sci. Tech., 22,1/2, 293-304.

Nicol, J.P., Benefield, L.D., Wetzel, E.D. and Heidman, J.A. (1988) "Activated sludge systems with biomass particle support structures", Biotechnol. Bioeng., 31, 682-695.

Norris, D.P., Parker, D.S., Daniels, M.L. and Owens, E.L. (1982) "Production of high quality trickling filter effluent without tertiary treatment", J. Wat. Pollut. Control Fed., 54, 1087-1098.

Nutt, S.G., Melcer, H. and Pries, J.H. (1984) "Two-stage biological fluidized bed treatment of coke-plant wastewater for nitrogen control", J. Wat. Pollut. Control Fed., 56, 7, 851-863.

Oliva, E., Jacquart, J.C. and Prevot, C. (1990) "Treatment of wastewater at the El Aguila brewery (Madrid, Spain) - Methanization in fluidized bed reactors", Wat. Sci. Tech., 22,1/2, 483-490.

Orr, P. and Lawty R. (1990) "Operating experience with large random packed biofilm reactors", Wat. Sci. Tech., 22,1/2, 203-214.

Parker, D.S. and Richards, T. (1986) "Nitrification in trickling filters", J. Wat. Pollut. Control Fed., 58, 896-902.

Polprasert, C. and Park, H.S. (1986) "Effluent denitrification with anaerobic filters", Wat. Res., 20, 8, 1015-1021.

Pujol, R. (1990) "Suivi du fonctionnement d'une station d'épuration associant physico-chimie et biofiltration : Metabief (France)", Wat. Sci. Tech., 22,1/2, 251-260.

Pujol, R. (1991) "L'épuration par biofiltration : premiers constats", Etude inter-agences n°2, CEMAGREF Lyon, France.

Racault, Y. (1990) "Treatment of distillery wastewater using an anaerobic downflow stationary fixed-film reactor: performance of a large plant in operation for four years", Wat. Sci. Tech., 22,1/2, 361-372.

Ravarini, P., Coutelle, J. and Damez, F. (1988) "L'usine de Dennémont - une unité de dénitrification-nitrification à grande échelle", T.S.M. L'eau, 83, 4, 235-240.

Richard, Y. and Partos, J. (1986) "Elimination biologique des nitrates en vue de la production d'eau potable. Bilan de fonctionnement de deux installations industrielles", T.S.M. L'eau, 3, 141-147.

Rogalla, F. and Payraudeau, M. (1988) "Tertiary nitrification with fixed biomass reactors", Wat. Supply, 6, 347-354.

Schlegel, S. (1988) "The use of submerged biological filters for nitrification", Wat. Sci. Tech., 20, 4/5, 177-187.

Seyfried, C.F. and Austermann-Haun, U. (1990) "Large scale anaerobic/aerobic treatment plants for molasses distillery, pectin-factory and wastes of starch factories", Wat. Sci. Tech., 22,1/2, 353-360.

Sutton, P.M., Shieh, W.K., Kos, P. and Dunning, P.R. (1981) "Dorr-Oliver's Oxitron system fluidized bed water and wastewater treatment process", in "Biological fluidized bed treatment of water and wastewater", P.F. Cooper and F. Atkinson Eds., Ellis Horwood, Chichester, England, 284-304.

BIOFILMS ASSOCIATED WITH HEALTH

T.R. NEU, H.C. VAN DER MEI, H.J. BUSSCHER
Laboratory for Materia Technica
University of Groningen
Antonius Deusinglaan 1
9713 AV Groningen
The Netherlands

1. Introduction

Biofilms associated with health can be approached from three different points of view (Costerton *et al.,* 1987). Firstly, biofilms can be distinguished which are directly associated with tissue surfaces in the body. These biofilms in the healthy body occur for example in the oral cavity both on teeth as well as on the oral mucosa, but also in the digestive tract and in the female genital tract. These "normal" biofilms may have a role in the prevention of certain infections (Embery *et al.,* 1991, Reid *et al.,* 1990). However, in these habitats pathogenic microorganisms are able to overgrow this "normal" biofilm. Further areas in the body where pathogenic biofilms may develop are the airway and urinary tract (see Table 1 for a summary).

The mechanisms by which a pathogenic microflora can overgrow the normal biofilm are manifold and include microbial cell surface compounds and excreted microbial compounds such as enzymes, bacteriocins or biosurfactants. The change from a normal bacterial flora to a sudden dominance of pathogenic organisms may be induced by changes in tissue surface chemistry, antibiotic therapy or age (Costerton *et al.,* 1987, Reid *et al.,* 1990). It has been suggested by Pratt-Terpstra *et al.,* (1989), that a biosurfactant producing *Streptococcus mitis*, an oral non-pathogenic microorganism, may play a role in preventing the adhesion of cariogenic mutans streptococci. A role of microbial biosurfactants in other parts of the body is not unlikely, though never studied vigorously up to date.

In most biofilms directly associated with tissue surfaces in the human body, the biofilms have to persist against shear forces due to periodically flushing or mechanical movement. In order to overcome these shear forces the micro-

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 21–34.

organisms developed strategies to adhere more tightly including the production of adhesive polymers and the use of fimbriae, fibrils and other surface appendages.

TABLE 1. Examples of microorganisms found in commensale, normal biofilms and their pathogenic counterparts.

Physiological environment	Normal microflora	Pathogenic microflora
skin	Corynebacteria Mycobacteria Streptococci Staphylococci Micrococci Propionibacteria	Candida Fungi
oral mucosa	Streptococci Micrococci Pneumococci Actinomyces Veillonella Peptostreptococci	Bacteroides Actinobacillus Fusobacterium Candida
digestive tract	Bacteroides Escherichia Streptococci	Vibrio Shigella
vagina	Lactobacilli Bacteroides Gardnerella Mobiluncus Candida	Escherichia

Secondly, there are biofilms associated with biomaterials implanted in the human body (Bisno and Waldvogel, 1989; Dankert *et al.,* 1986; Gristina, 1987; Jaques *et al.,* 1987). Medical biomaterials are made from a variety of different materials with a wide range of mechanical and surface properties. The materials include rubber, polymers, metals and ceramics are used for different types of

implants such as prostheses, grafts and catheters. The crucial property of a biomaterial is its biocompatibility. By definition a biocompatible material is a material which does not lead to an acute or chronic inflammatory response and that does not prevent a proper differentiation of implant surrounding tissues (Williams, 1987). It is very difficult to implant a biomaterial totally sterile and therefore they become frequently colonized by microorganisms. However, other indirect entry paths for microorganisms are also possible.

Thirdly, biofilms can be distinguished which are not directly associated with health. These biofilms occur for example in water distribution systems or in the cooling towers of air conditioning systems (Flemming and Geesey, 1991; Howsam, 1990). There appears to be a general consensus in biomaterials research that there is not yet a material which will not eventually become colonized by microorganisms.

However, there is also a consensus that it should be possible to reduce the adhesive forces that keep the biofilm attached to a substratum or the cohesive forces within a biofilm (Mozes *et al.,* 1987; Büchs *et al.,* 1988). Such an approach puts major emphasis on the interaction of the initially colonizing strains with the substratum. Furthermore, this approach would allow easier removal of the biofilm despite its formation. Also, when the physical environment of the biofilm under consideration would include shear, a natural removal might be feasible.

This review will concentrate on biofilms occurring on biomaterials implanted in the human body, with special emphasis on the formation of a biofilm on biomaterials and the impact of excreted microbial molecules as anti-adhesive compounds. Finally, multispecies bacterial biofilms and mixed procaryotic - eucaryotic biofilms will be elaborated upon and a case study on mixed biofilms on silicone voice prostheses will be presented.

2. Biofilm formation on biomaterials

Whether or not a biomaterial is implanted totally inside the human body or partly in connection with the outside, it has been argued that it is inevitable that a microbial infection will occur sooner or later. Gristina has called biomaterial implants "a microbial time bomb", the most commonly isolated strains being *Escherichia coli*, staphylococci or pseudomonas species. The initial events after implantation of a biomaterial include the formation of a conditioning film and a subsequent "race for the surface" (Gristina *et al.,* 1989a) between tissue cells and microorganisms. If this race is won by the tissue cells, a long term patency of the implant is generally insured.

The conditioning film will be built up by molecules originating from the

different body fluids surrounding the biomaterial such as saliva, mucus, serum or blood (Gristina *et al.*, 1991). Depending on the surface properties of the biomaterial different molecules will adsorb to the biomaterial. In addition, the molecules may adsorb in various ways as different parts of a macromolecule may have a different hydrophobicity and charge. Thus the three-dimensional orientation of a molecule at an interface can be totally different on various biomaterials as pointed out by Neu and Marshall (1990) and therewith affect the interaction of cells and microorganisms with the biomaterial.

In order to colonize biomaterials, microorganisms have to be transported to the surface. This process may be active or passive. For motile bacteria and microorganisms actually growing "into" the body along an interface, as for example in urinary tract infections, it might be an active process. If the microorganisms are transported with the body fluids it is evidently a passive process.

During the initial adhesion phase, physicochemical properties of the microbial cell surface as charge and hydrophobicity are important and determine whether the cell can adhere to the surface (Rouxhet and Mozes, 1990). The significance of microbial cell surface hydrophobicity in adhesion to biomaterials was recently discussed in detail by Klotz (1990) emphasizing that most hydrophobic bacteria are not virulent or pathogenic to the normal host, but that they will become involved in infections when host barriers are breached due to the insertion of a biomaterial.

After the initial adhesion step, the microorganisms attach to the surface involving amongst other factors the production of microbial polymers (Neu and Marshall, 1990), mediating the final strength by which the biofilm will be attached to the surface. At this stage it is important to realize that the final biofilm is only attached as strongly as its weakest chain which should preferentially be represented by the microorganisms and their polymers in direct contact with the interface.

After the attachment phase the microorganisms may start to grow at the interface and in a colonization step eventually will form microcolonies and develop into a biofilm. The microorganisms in the biofilm are immobilized in a polymer matrix which has a significant role in biofilm processes (Neu, 1992). Further colonization can involve microbial cell-cell interactions which may be observed after initial adhesion by pioneering microorganisms (Dworkin, 1991).

The final phase in biofilm persistance are removal processes. For biomaterials this includes active detachment of single microbial cells, shear related removal and sloughing (spontaneous detachment of larger parts of the biofilm). In studies on the sloughing of biofilms it was found that anaerobic gas production by bacteria and extracellular polymers of bacteria are involved in the detachment from surfaces (Bryers, 1988).

3. Biofilm matrix polymers and microbial biosurfactants

Microbial surface polymers involved in the interaction of cells with interfaces are mainly polysaccharides (Christensen, 1989, Jann and Jann, 1990a; Neu, 1992). However, also proteins may be present in the form of S-layers, protein capsules, fimbriae or fibrils (Jann and Jann, 1990b).

In biofilms, microbial exopolymers build up the biofilm matrix. This matrix represents a three dimensional network which is responsible for the mechanical properties of the biofilm and which is involved in the chemical processes between the bulk phase and the biofilm as well as within the biofilm. The function of polysaccharides in biofilms on biomaterials are wide spread and are listed in Table 2.

TABLE 2. Possible functions of bacterial polysaccharides in infections related to biomaterials (Neu, 1992).

Form of polysaccharide	Role in health related biofilms
Intracellular	Energy storage
Cell surface bound	Reversible adhesion
	Microbial aggregation
	Bacterial capsule antigen
	Protection against phagocytosis
Matrix polymer	Microcolony formation
	Cryptic growth
	Protection from antibodies
	Protection from complement system
	Protection from antibiotics
	Ionic interactions
Released polymer	Bacterial antigen
	Microbial detachment

By employing scanning confocal laser microscopy fully hydrated microbial biofilms have been visualized. It was found that, depending on the species, a biofilm may show a certain cell density at the attachment surface and become more diffuse in the outer regions. However, with other species the opposite arrangement has been observed. In addition, it could be demonstrated that a biofilm does not represent a closed layered system, but that it shows an open architecture. A network of channels connecting the outer layers with the basal

layers of the biofilm allows the exchange of gas and nutrients (Lawrence *et al.,* 1991).

The biofilm mode of growth of infectious microorganisms is especially troublesome in health related problems because antibiotics are not very effective against microorganisms in biofilms and the concentration to achieve the same effect compared to planktonic cells is several magnitudes higher. The antibiotic resistance of *Pseudomonas aeruginosa* biofilm and planktonic cells was investigated in detail by Nickel *et al.,* (1985) and it was found that bacteria in old biofilms were the most resistant compared to bacteria in young biofilms and planktonic cells (Anwar *et al.,* 1989). By exposing Pseudomonas bacteria to Ca^{2+} ions they showed a higher resistance against tobramycin (Hoyle and Costerton, 1989). A combination of two antibiotics, tobramycin and piperacillin, resulted in a enhanced killing of biofilm bacteria (Anwar *et al.,* 1990).

Comparative studies with several biomaterials and coagulase-negative staphylococci using a range of different antibiotics could also demonstrate a higher resistance of sessile bacteria (Gristina *et al.,* 1989b). Although it is not exactly clear why sessile cells are less sensitive, it has been suggested that this is due to the shielding acting of the polysaccharides and cell clumps in the biofilm, as visualized by confocal laser microscopy.

Finally, microbial compounds which can be excreted and possess surface active properties may have a determinant influence on the ultimate microbial composition of the biofilm. An *in vitro* study on oral bacteria showed that excreted substances from *Streptococcus mitis* decreased the adhesion of the cariogenic strain, *Streptococcus mutans*, on glass. Furthermore, the surface active character of this compound could be demonstrated (Pratt-Terpstra *et al.,* 1989). Flow chamber experiments with bacteria from the periodontal pocket indicated that pre-conditioning of glass with *Streptococcus cricetus* reduced the deposition rate of *Prevotella intermedia* (Cowan and Busscher, submitted), possibly by leaving "microbial footprints" (Neu and Marshall, 1991, Neu, in press). Surface active compounds were also involved in stimulating the desorption of *Streptococcus thermophilus*, a dairy organism, from glass surfaces. The surface bound molecules produced by the adhering cells themselves prevented the later deposition of freshly cultured cells to this interface (Busscher *et al.,* 1990). The above examples point out that there may be a role for microbial biosurfactants in establishing multispecies biofilms.

4. Multispecies biofilms

Biofilms in nature are built up by mixed populations of different microorganisms.

However, apart from a few early studies (Dias *et al.,* 1968, Maigetter and Pfister, 1974, Veldkamp and Janasch, 1972) mixed species biofilms were investigated mainly in the last few years. In binary bacterial biofilms it could be shown that adhesion is influenced by the presence of another species, the species combination, the surface properties and the sequence of adhesion (McEldowney and Fletcher, 1987). Similar results are reported by authors studying the competitive adherence of bacteria to nasal epithelial cells (Bibel *et al.,* 1983). In another report on binary population biofilms it was demonstrated that the establishment of a microorganism is a function of its growth rate (Banks and Bryers, 1991). Oppositely, it was reported for a *Klebsiella pneumoniae* - *Pseudomonas aeruginosa* binary biofilm, that both species do not influence each other (Siebel and Characklis, 1991). When true multispecies biofilms are investigated the results were even more differentiated as described for the colonization of glass by using four different bacterial strains (Cowan *et al.,* 1991).

A few years ago it was still not possible to study the distribution of a certain bacterial population within a biofilm. The development of oligonucleotide probes and the use of the polymerase chain reaction made it possible to detect even a single bacterial cell within a mixed biofilm. Now for quick identification of specific bacterial populations within a biofilm, fluorescent dye-labeled oligonucleotide probes are available (Amann *et al.,* 1990, Amann *et al.,* 1992).

Biofilms in nature represent a complex sytem in which bacteria may dominate. However, natural biofilms are always subject to grazing by eucaryotic organisms. In addition, true mixed procaryotic - eucaryotic biofilms can also be found. This situation is typical for the medical field where often mixed bacteria - yeasts biofilms are described in colonizing tissues as well as biomaterials. Only a few reports exist on mixed procaryotic - eucaryotic biofilms in the medical/ biomaterial field (see also section 5.). In most cases the studies concentrated on the isolation of the microorganisms to detect potential pathogens as for example in catheter-related vascular infections (Sherertz *et al.,* 1990). Other investigations described the interaction of bacteria with eucaryotyic cells as in the case of ocular inflammations caused by bacteria and amoebae (Johns, 1991).

5. Case study on mixed bacteria - yeasts biofilms

A mixed bacteria - yeasts biofilm can be found on silicone voice prostheses. Silicone voice prostheses are implanted in patients after laryngectomy. The prosthesis is inserted in a shunt between the tracheostoma, through which the patients have to breath, and the upper digestive tract. Thus the prostheses are located in an unsterile environment and become rapidly colonized by

microorganisms which results in the frequent replacement of the prostheses.

A mixed bacteria-yeasts biofilm could be demonstrated on explants both by scanning electron microscopy (Neu *et al.,* submitted a) and by isolating the microorganisms (Neu *et al.,* submitted b). Scanning electron microscopy showed a dense biofilm (see Fig. 1) covering the entire prostheses already after several weeks. If the microorganisms colonize the inner parts of the valve (see Fig. 2) it will lead to the failure of the prosthesis. Details of the mixed biofilm are shown in Fig. 3 revealing the close association of different yeast forms and bacteria.

The identification of the microorganisms revealed about two third of the species as bacteria, mainly streptococcal species and one third as yeasts, mainly candida species. Cell surface characterization showed that only a few bacteria have fimbriae; however, most of the strains possess a ruthenium red stained layer.

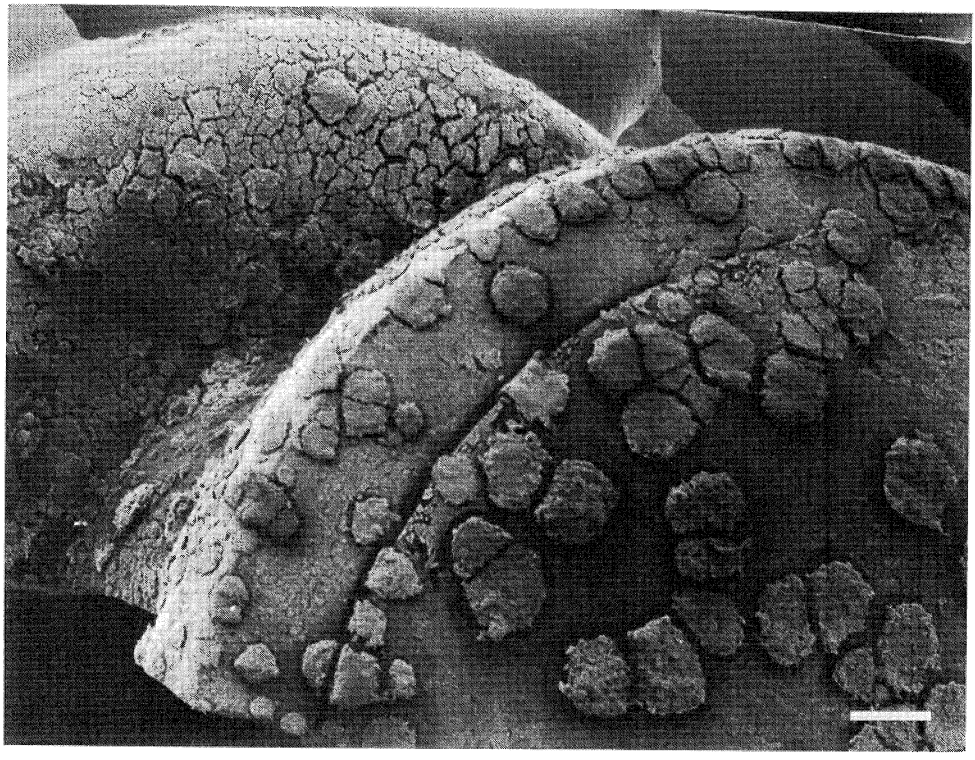


Figure 1. Scanning electron micrograph of the "Groningen Button" silicone voice prosthesis covered by a mixed biofilm consisting of bacteria and yeasts (bar 1.5 mm).

Figure 2. Scanning electron micrograph of the valve region colonized by microorganisms eventually leading to the failure of the prosthesis (bar 100 μm).

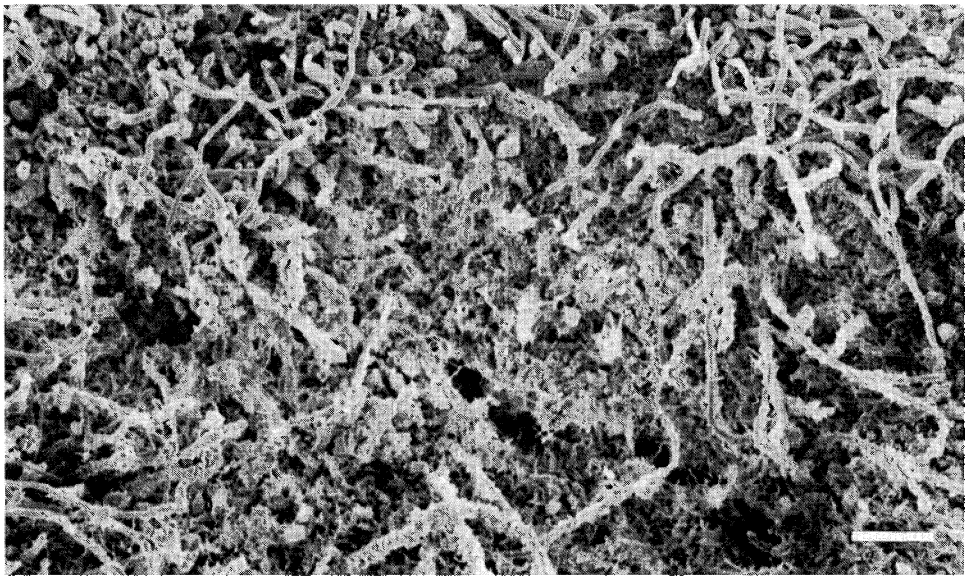


Figure 3. Scanning electron micrograph demonstrating the mixed bacteria-yeasts biofilm on a silicone voice prosthesis (bar 25 μm).

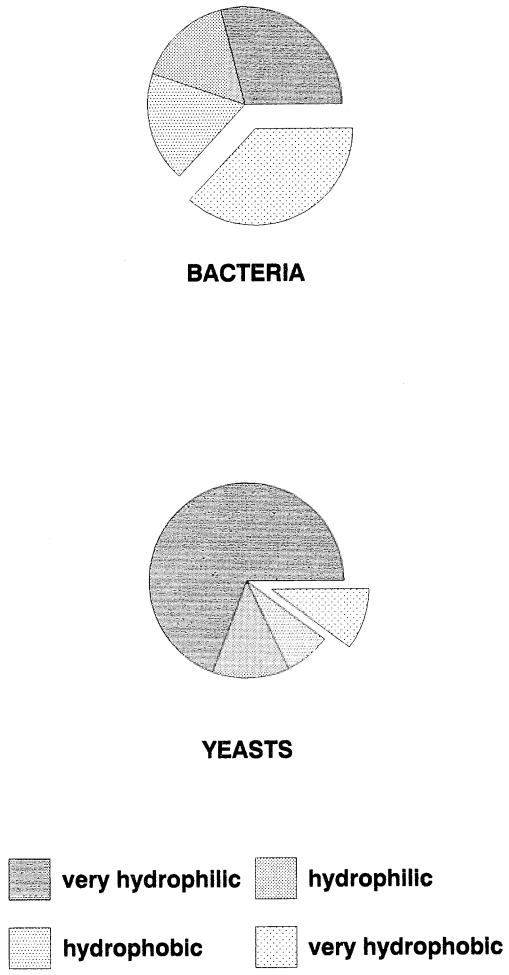


Figure 4. Comparison of cell surface hydrophobicities of bacteria and yeasts isolated from silicone voice prostheses determined by microbial adhesion to hydrocarbons.

Cell surface hydrophobicity as by microbial adhesion to hydrocarbons varied (Neu *et al.,* submitted b). Hydrophobicity values of the bacteria were in general much higher than those of the yeasts (see Fig. 4). On a purely physicochemical basis this is hard to understand because it would be expected that only hydrophobic cells would adhere to the hydrophobic silicone rubber and not also hydrophilic cells. In oral microbiology mixed bacteria-yeast biofilms have been described in relation to denture stomatitis. The initial colonization by bacteria of a denture turned out to be a prerequisite for the subsequent adhesion by *Candida albicans* (Verran and Motteram, 1987).

Thus we hypothetically explain the colonization of the silicone voice prosthesis by hydrophobic bacteria and by hydrophilic yeasts as follows. The initially adhering hydrophobic bacteria may precondition the silicone material with a biosurfactant to render it more hydrophilic so that the hydrophilic yeast cells are subsequently able to colonize the prostheses.

This hypothesis is under further investigation and, when proven, may affect our way of approaching the formation mechanism of complex biofilms in health related problems and other areas.

6. References

Amann, R.I., Krumholz, L. and Stahl, D.A. (1990) 'Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology', J. Bacteriol. 172, 762-770.

Amann, R.I., Stromley, J., Devereux, R., Key, R. and Stahl, D.A. (1992) 'Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms', Appl. Environ. Microbiol. 58, 614-623.

Anwar, H., Dasgupta, M., Lam, K. and Costerton, J.W. (1989) 'Tobramycin resistance of mucoid *Pseudomonas aeruginosa* biofilm grown under iron limitation', J. Antimicrob. Chemother. 24, 647-655.

Anwar, H., Dasgupta, M.K. and Costerton, J.W. (1990) 'Testing the susceptibility of bacteria in biofilms to antibacterial agents', Antimicrob. Agents Chemother. 34, 2043-2046.

Banks, M.K. and Bryers, J.D. (1991) 'Bacterial species dominance within a binary culture biofilm', Appl. Environ. Microbiol. 57, 1974-1979.

Bibel, D.J., Aly, R., Bayles, C., Strauss, W.G., Shinefield, H.R. and Maibach, H.I. (1983) 'Competitive adherence as a mechanism of bacterial interference', Can. J. Microbiol. 29, 700-703.

Bisno, A.L. and Waldvogel, F.A. (1989) Infections Associated with Indwelling Medical Devices, American Society of Microbiolgoy, Washington.

Bryers, J.D. (1988) 'Modelling biofilm accumulation', in M.J. Bazin and J.I. Prosser (eds.), Physiological Models in Microbiology, CRC Press, Boca Raton, pp. 109-144.

Büchs, J., Mozes, N., Wandrey, C. and Rouxhet, P.G. (1988) 'Cell adsorption control by culture conditions', Appl. Microbiol. Biotechnol. 29, 119-128.

Busscher, H.J., Bellon-Fontaine, M-N., Mozes, N., Van der Mei, H.C., Sjollema, J., Cerf, O. and Rouxhet, P.G. (1990) 'Deposition of *Leuconostoc mesenteroides* and *Streptococcus thermophilus* to solid substrata in a parallel flow cell', Biofouling 2, 55-63.

Christensen, B.E. (1989) 'The role of extracellular polysaccharides in biofilms', J. Biotechnol. 10, 181-202.

Costerton, J.W., Cheng, K-J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. (1987) 'Bacterial biofilms in nature and disease', Ann. Rev. Microbiol. 41, 435-464.

Cowan, M.M., Warren, T.M. and Fletcher, M. (1991) 'Mixed-species colonization of solid surfaces in laboratory biofilms', Biofouling 3, 23-34.

Cowan, M.M. and Busscher, H.J. () 'Flow chamber study of the adhesion of *Prevotella intermedia* to glass after preconditioning with Streptococcus species', (submitted for publication to FEMS Microbiology Letters)

Dankert, J., Hogt, A.H. and Feijen, J. (1986) 'Biomedical polymers: Bacterial adhesion, colonization, and infection', CRC Crit. Rev. Biocomp. 2, 219-301.

Dias, F.F., Dondero, N.C. and Finstein, M.S. (1968) 'Attached growth of Sphaerotilus and mixed populations in a continuous flow apparatus', Appl. Microbiol. 16, 1191-1199.

Dworkin, M. (1991) Microbial Cell-Cell Interactions, American Society for Microbiology, Washington.

Embery, G., Bagg, J. and Wassall, M. (1991) 'Bacterial adhesion to the soft a n d hard tissues of the oral cavity', Biofouling 3, 223-235.

Flemming, H-C. and Geesey, G.G. (1991) Biofouling and Biocorrosion in Industrial Water Systems. Springer Verlag, Heidelberg.

Gristina, A.G. (1987) 'Biomaterial-centered infection: Microbial adhesion versus tissue integration', Science 237, 1588-1595.

Gristina, A.G., Naylor, P.T. and Myrvik, Q.N. (1989a) 'The race for the surface: Microbes, tissue cells, and biomaterials', in L. Switalski, M. Höök and E. Beachey (eds.), Molecular Mechanisms of Microbial Adhesion, Springer, New York, pp. 177-211.

Gristina, A.G., Jennings, R.A., Naylor, P.T., Myrvik, Q.N. and Webb, L.X. (1989b) 'Comparative *in vitro* antibiotic resistance of surface-colonizing coagulase-negative Staphylococci', Antimicrob. Agents Chemother. 33, 813-816.

Gristina, A.G., Naylor, P.T., Meador, T.L. and Myrvik, Q.N. (1991) 'Biomaterial

surface domains: Bacterial versus tissue cells', in M. Bender (ed.), Interfacial Phenomena in Biological Systems. Marcel Dekker, New York, pp. 105-136.

Howsam, P. (1990) Microbiology in Civil Engineering, Chapman & Hall, London.

Hoyle, B.D. and Costerton, J.W. (1989) 'Transient exposure to a physiologically-relevant concentration of calcium confers tobramycin resistance upon sessile cells of *Pseudomonas aeruginosa*', FEMS Microbiol. Lett. 60, 339-342.

Jann, K. and Jann, B. (1990a) Bacterial Capsules, Current Topics in Microbiology and Immunology 150. Springer Verlag, Heidelberg.

Jann, K. and Jann, B. (1990b) Bacterial Adhesins. Current Topics in Microbiology and Immunology 151. Springer Verlag, Heidelberg.

Jaques, M., Marrie, T.J. and Costerton, J.W. (1987) 'Review: Microbial colonization of prosthetic devices', Microb. Ecol. 13, 173-191.

Johns, T. (1991) 'Interactions of bacteria and amoebae with ocular biomaterials', Cells & Materials 1, 129-139.

Klotz, S.A. (1990) 'Role of hydrophobic interactions in microbial adhesion to plastics used in medical devices', in R.J. Doyle and M. Rosenberg (eds.) Microbial Cell Surface Hydrophobicity, American Society of Microbiology, Washington, pp. 107-136.

Lawrence, J.R., Korber, D.R., Hoyle, B.D., Costerton, J.W. and Caldwell, D.E. (1991) 'Optical sectioning of microbial biofilms', J. Bacteriol. 173, 6558-6567.

Maigetter, R.Z. and Pfister, R.M. (1974) 'A mixed bacterial population in a continuous culture with and without kaolinite', Can. J. Microbiol. 21, 173-180.

McEldowney, S. and Fletcher, M. (1987) 'Adhesion of bacteria from mixed cell suspension to solid surfaces', Arch. Microbiol. 148, 57-62.

Mozes, N., Marchal, F., Hermesse, M.P., Van Haecht, J.L., Reuliaux, L., Leonard, A.J. and Rouxhet, P.G. (1987) 'Immobilization of microorganisms by adhesion: Interplay of electrostatic and nonelectrostatic interactions', Biotechnol. Bioeng. 30, 439-450.

Neu, T.R. and Marshall, K.C. (1990) 'Bacterial polymers: Physicochemical aspects of their interactions at interfaces', J. Biomaterials Applications 5, 107-133.

Neu, T.R. and Marshall, K.C. (1991) 'Microbial footprints - a new approach to adhesive polymers', Biofouling 3, 101-112

Neu, T.R. (1992) 'Polysaccharide in Biofilmen', in P. Präve, M. Schlingman, W. Crueger, K. Esser, R. Thauer and F. Wagner (eds.), Jahrbuch Biotechnologie Band 4. Carl Hanser Verlag, München, pp. 73-101.

Neu, T.R. (1992) 'Microbial "footprints" and the general ability of microorganisms to label interfaces', Canadian J. Microbiol. (in press)

Neu, T.R., Dijk, F., Verkerke, B., Van der Mei, H.C. and Busscher, H.J. () 'Scanning electron microscopy study of biofilms on silicone voice prostheses',

(submitted for publication to Cells and Materials a)

Neu, T.R., Herrmann, I.F., Schutte, H.K., Van der Mei, H.C. and Busscher, H.J. () 'Isolation and characterization of microorganisms from silicone voice prostheses' (submitted for publication to Journal of Clinical Microbiology b)

Nickel, J.C., Ruseka, I., Wright, J.B. and Costerton, J.W. (1985) 'Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material', Antimicrob. Agents Chemother. 27, 619-624.

Pratt-Terpstra, I.H., Weerkamp, A.H., Busscher, H.J. (1989) 'Microbial factors in a thermodynamic approach of oral streptococcal adhesion to solid substrata', J. Colloid Interface Sci. 129, 568-574.

Reid, G., Bruce, A.W., McGroarty, J.A., Cheng, K-J. and Costerton, J.W. (1990) 'Is there a role for Lactobacilli in prevention of urogenital and intestinal infections', Clinical Microbiology Reviews 3, 335-344.

Rouxhet, P.G. and Mozes, N. (1990) 'Physical chemistry of the interface between attached microorganisms and their support', Wat. Sci. Tech. 22, 1-16.

Sherertz, R.J., Raad, I.I., Belani, A., Koo, L.C., Rand, K.H., Pickett, D.L., Straub, S.A. and Fauerbach, L.L. (1990) 'Three-year experience with sonicated vascular catheter cultures in a clinical microbiology laboratory', J. Clin. Microbiol. 28, 76-82.

Siebel, M.A. and Characklis, W.G. (1991) 'Observations of binary population biofilms', Biotechnol. Bioeng. 37, 778-789.

Veldkamp, H. and Jannasch, H.W. (1972) 'Mixed culture studies with the chemostat', J. Appl. Chem. Biotechnol. 22, 105-123.

Verran, J. and Motteram, K.L. (1987) 'The effect of adherent oral streptococci on the subsequent adherence of *Candida albicans* to acrylic *in vitro*', J. Dent. 15, 73-76.

Williams, D.F. (1987) Definitions in Biomaterials. Progress in Biomedical Engineering 4. Elsevier, Amsterdam.

INTRODUCTION TO BIOFILMS IN THE FOOD INDUSTRY

J.T. HOLAH AND L.R. KEARNEY
Campden Food and Drink Research Association
Chipping Campden
Glos GL55 6LD
United Kingdom

1. Introduction

Of major importance in food processing is the freedom of microbial (spoilage and pathogenic microorganisms) and foreign body contamination in the finished product. Such contamination may lead to product recalls with their associated adverse publicity resulting in loss of customers, sales and profits. If regulatory requirements have been infringed, fines, sanctions or ultimately site closure or loss of export licence may ensue.

Contamination in food products may arise from 4 main sources; the constituent raw materials, surfaces, people (and other animals) and the air. Control of the raw materials is addressed for example, by specifications, positive release of materials after sampling, QA/QC procedures and supplier audits, and is the only non environmental contamination route. Food may pick up contamination as it is moved across product contact surfaces or if it is touched or comes into contact with people (food handlers) or other animals (pests). The air acts as both a source of contamination, i.e. from outside the processing area or as a transport medium e.g. moving contamination from non product to product contact surfaces.

In other scientific disciplines, biofilms are envisaged as biological growths on surfaces consisting of higher organisms (sometimes), a multitude of microbial cells and extracellular polymers which develop with time. However, in the food processing industries, the time dimension for biofilm development is usually relatively short. Production lines in many food industries may operate for as little as less than an hour, while others may run for several days. In addition, the cleaning regimes on most sites are coordinated to coincide with the varying production intervals, or with what is deemed practical to comply with adequate hygienic standards throughout the processing site, and as such, time for biofilm formation may be limited. For the food processing industry, therefore, the term biofilm is more associated with the attachment and growth of microorganisms on surfaces rather than the development of thick biological films over long time periods. The type of microbial species present is also extremely important and whilst relatively high levels of non-spoilage or non-pathogenic microorganisms on surfaces may be tolerated, the presence of food pathogens e.g. *Salmonella* spp or *Listeria* spp would generally not be acceptable. Nevertheless food processing environments have an abundance of exposed surfaces upon which microbes may attach, grow, develop into microcolonies and possibly form a surface covering in excess of 10^7 cells/cm^2 which constitutes a 'typical' biofilm.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 35–41.

TABLE 1. Summary of literature reports on microbial adhesion and biofilms in food processing environments

Product	Time	Count	Method	Reference
Raw Milk	20h		S.E.M.	Zoltai *et al.* (1981)
Raw Milk (inoculated)	2-6h 12h	0 10^4/cm²	Swab	Bouman *et al.* (1984)
Pasteurised milk (inoculated)	2-6h 12h	Organisms present 10^6/cm²		
Milk	5 days	8.13×10^3/cm² Mesophiles (ss) 3.47×10^4/cm² Mesophiles (rubber) 8.51×10^3/cm² Psychrotrophs (ss) 8.55×10^4/cm² Psychrotrophs (rubber)	Squeegee rinse	Lewis & Gilmour (1987)
Baked beans transport belt	2-4h 6-8h 12h 16h	2.06×10^6 /cm² 1.07×10^7 /cm² $>1.67 \times 10^7$ /cm² $>4.30 \times 10^7$ /cm²	DEM	Holah *et al.* (1989)
Egg glaze bath	0-2h 2-4h 4-6h 6-8h	8.04×10^4 /cm² 2.00×10^4 /cm² 9.00×10^4 /cm² 1.70×10^6 /cm²		
Fish filleting	0-2h 2-4h 4-6h 6-8h	4.00×10^4 /cm² 2.30×10^4 /cm² 1.30×10^4 /cm² 3.35×10^3 /cm²		
Buttermilk in margarine production	0-2h 2-4h 4-6h 6-8h	2.26×10^4 /cm² 1.84×10^4 /cm² 1.76×10^4 /cm² 8.07×10^5 /cm²		

Biofilm development in these environments can have detrimental effects on the microbial status of the food. The presence of biofilms on surfaces harbouring many bacteria, including pathogens, can contaminate the food through direct contact or indirectly by vectors such as personnel, pests, air movement and cleaning systems. As a consequence, there is an increased chance of food spoilage that may lead to reduced shelf life and an increase in the risk of food poisoning from pathogens.

Provided that the process environment and production equipment have been hygienically designed, cleaning and disinfection, or referred to together as sanitation, is the major day to day control of the 'surface' route of food product contamination. When undertaken correctly, sanitation programmes have been shown to be cost effective, easy to manage and if diligently applied, can reduce the risk of microbial and foreign body contamination. Given the intrinsic demand for high standards of hygiene in the production of short shelf-life chilled foods, together with pressure from customers, consumers and legislation for ever increasing hygiene standards, sanitation demands the same degree of attention as any other key process in the manufacture of safe and wholesome chilled foods. In addition to removing microorganisms and/or material conducive to microbial growth, sanitation programmes extend the life of and prevent damage to equipment and services, provide a safe and clean working environment for employees, boost morale and productivity and present a favourable image to customers and the public.

2. Food Industry Biofilms

The development and control of biofilms has been widely studied in many diverse fields, including medical (Costerton, 1984; Marrie and Costerton 1984; Absolom, 1988; Conway and Ronald, 1988), water treatment (Bryers and Characklis, 1990) and ore extraction (Bryers, 1990). In contrast relatively few studies have been published on biofilms in the food industry.

TABLE 2. Microbial levels on vegetable processing contact surfaces

Area	Coupon Exposure (hours)	Count/cm² on attached plate	Count/Swab (25cm²)
Raw vegetable			
Carrot Line	5	9.60×10^4	1.76×10^6
Potato Line	5	5.43×10^4	3.81×10^6
Cooked vegetable			
Carrot Line	6	3.63×10^4	4.42×10^6
Potato Line	6	2.50×10^4	7.80×10^2
Bean filling	24	1.27×10^5	3.97×10^8

Literature reports on microbial adhesion to surfaces in food processing sites is reviewed in Table 1. In the dairy industry, Zoltai *et al.* (1981) used scanning electron microscopy to demonstrate the adhesion of bacteria to stainless steel

chips secured to the inside of a milk storage tank. Bouman *et al.* (1982) also used scanning electron microscopy to illustrate the attachment of microbes to the surface of a pilot plant milk pasteuriser. Enumerating surface contamination levels using a swab technique, they found higher microbial levels on the plates in the pasteurised section than the raw milk section. Similar results were obtained by Driessen *et al.* (1984) in laboratory experiments, which they attributed to growth inhibiting compounds in the raw milk.

Lewis and Gilmour (1987) investigated the adhesion of natural milk flora to transfer pipes composed of stainless steel and rubber. They found that quantitatively and generically the adherent population on the two surfaces were not significantly different. This is in contrast to a review by Fletcher (1991) which details several reports of substratum influences on microbial attachment. Lewis and Gilmour (1987) also found that the microbial constitution of the milk differed from that of the adherent population.

TABLE 3. Microbial levels on processed can lines

Area	Coupon exposure (hours)	Count/cm² on attached plate	Count/Swab (25cm²)
Processed can line 1	24	3.42×10^4	2.55×10^2
Processed can line 2	24	4.25×10^5	4.55×10^2
Processed can line 3	6	8.35×10^4	1.38×10^3
	16	4.40×10^4	ND
Beneath can line	20	1.53×10^5	
Processed can line 4	6	1.55×10^4	4.65×10^2
	16	6.70×10^4	ND
	24	1.65×10^4	
Waste can discharge area	4	1.73×10^6	1.11×10^8
	24	($>3.50 \times 10^7$)	

Microbial adhesion to various food processing surfaces was also studied by Holah *et al.* (1989). This involved attaching stainless steel plates, or coupons, to different product lines for varying time intervals, subsequent staining with acridine orange and examination using direct epifluorescent microscopy (DEM). The results of the trials showed microcolonies formed on an egg glaze and a buttermilk line, while multilayered biofilms developed after 8 hours on a baked

bean line.

3. Current Work

Investigations into the development of food processing environmental surface biofilms is now continuing at Campden Food and Drink Research Association under the auspices of a 3 year Ministry of Agriculture Fisheries and Food research project. The aim of this project is to identify locations in factories where biofilms may develop, elucidate the critical factors promoting and preventing their formation, and establishing methods for their control.

The results of some recent factory trials are shown in Table 2. To determine microbial attachment to surfaces and biofilm development, stainless steel plates (10cm x 4cm) were attached to surfaces, stained with acridine orange on removal, and examined using an epifluorescent microscope linked to an Optomax V image analyser (Synoptics Image Processing Systems, Cambridge). Surface contamination was also enumerated by a traditional swabbing method.

Table 2 shows microbial accumulation on surfaces in vegetable processing environments. Over the time intervals studied, no true biofilm development was observed, but most of the results showed counts in excess of $10^4/cm^2$.

Microbial levels on processed can lines was also monitored (Table 3). The food process lines examined were arbitrarily designated lines 1 to 4. Along these lines microbial counts in excess of $10^4/cm^2$ development on the stainless steel plates during relatively short periods of operation, but no true biofilms were observed. Low microbial levels along these areas are critical as wet process can lines have been identified as one of the major causes of leaker spoilage of canned foods (Thorpe and Everton 1968, Put *et al.* 1980).

TABLE 4. Microbial levels on a navy bean blancher extractor

	Time (hours)	% Coverage of Plate	Count/Swab ($25cm^2$)
Occasion 1	24	26.51	1.96×10^8
Occasion 2	24	23.79	3.20×10^7
	24	20.19	
	48	66.34	
	72	85.24	

The stainless steel plate attached to the waste process can area, an automatic device which diverts unsuitable cans to a waste bin, showed multilayered biofilm development after 24 hours exposure. The plate in this area was continually subjected to water trickling from the post process can line above it. This moisture level, combined with the possible leakage of nutrients from damaged cans could contribute to the development of biofilms in this area. Although not

in direct contact with food or processed cans, biofilms in this area would potentially be a source of microbial spread through the factory by personnel, air movement or cleaning systems.

The build up of biofilms in a blancher extractor at a point a few meters from its exhaust was also examined on two separate occasions at one of the factory sites. Table 4 shows how rapidly multilayered biofilms developed in this environment. Biofilms associated with air extraction units are of concern in food production environments as spoilage and/or pathogenic microorganisms in aerosols may gain ingress to the factory via close by or down wind factory air intakes. The moist and warm atmosphere of the extractor provides ideal growth conditions for many organisms including *Legionella* spp which can survive in biofilms and aerosols (Lee and West 1991). If the extractor harboured *L.pneumophilia*, its exhausts to the environment could represent a potential health hazard.

Our results show that the microbial levels in surfaces can accumulate relatively rapidly in a variety of locations in food processing environments. However, at this early stage, no final conclusions on factors influencing their proliferation will be established until the survey of food processing sites has been completed in 1994. Given the increasing concern over food hygiene issues, it is hoped that other workers will also actively pursue studies in the food processing field.

REFERENCES

Bouman, S., Lund, D.B., Driessen, F.M. and Schmidt, D.G. (1982) Growth of thermoresistant streptococci and deposition of milk constituents on plates of heat exchangers during long operation times. *Journal of Food Protection* **45**, 806-812

Carson, M.O., Lillard, H.S. and Hamdy, M.K. (1987) Transfer of firmly attached P-*Salmonella typhimurium* from raw poultry skin to other surfaces. *Journal of Food Protection* 50, 327-329

Characklis, M.G. (1990) Biofilm processes. In *Biofilms*, Eds W.G. Characklis and K.C. Marshall, John Wiley and Sons Inc. USA 195-232

Charlton, B.R., Kinde, H., and Jensen, L.H. (1990) Environmental Survey for *Listeria* species in California milk processing plants. *Journal of Food Protection* 53, 198-201

Frank, J.F and Koffi, R.A. (1990) Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitisers and heat. *Journal of Food Protection* 53, 550-554

Holah, J.T., Betts, R.P., and Thorpe, R.H. (1989) The use of epifluorescent microscopy to determine surface hygiene. *International Biodeterioration* **25**,

147-153

Le Chevallier, M.W., Cawthon, C.D. and Lee, R.G. (1988) Inactivation of Biofilm Bacteria. *Applied and Environmental Microbiology* 54, 2492-2499

Lee, S-H., and Frank, J.F. (1991) Inactiviation of Surface Adherent *Listeria monocytogenes.* Hypochlorite and heat. *Journal of Food Protection* 54, 4-6

Lee, J.V. and West, A.A. (1991) Survival and growth of *Legionella* species in the environment. *Journal of Applied Bacteriology Symposium Supplement* 70, 121 S - 129 S

Lewis, S.J. and Gilmour A. (1987) Microflora associated with the internal surface of rubber and stainless steel milk transfer pipeline. *Journal of Applied Bacteriology* 62, 327-333

Marrie, T.J. and Costerton J.W. (1984) Morphology of bacterial attachment to cardiac pacemaker leads and power packs. *Journal of Clinical Microbiology* 19, 911-914

Put, H.M.C., Wituoet, H.J., and Warner, W.R. (1980) Mechanisms of microbiological leaker spoilage of canned foods: Biophysical aspects. *Journal of Food Protection* 43, 488-497

Thorpe, R.H. and Everton, J.R. (1968) Post-Process sanitation in canneries. *Technical Manual No.1,* CFDRA, Chipping Campden, UK.

Zoltai, P.T., Zottola, E.A. and Mckay, L.L. (1981) Scanning electron microscopy of microbial attachment to milk contact surfaces. *Journal of Food Protection* 44, 204-208

Chapter 2

ATTACHMENT

PHYSICO-CHEMICAL ASPECTS OF ADHESION

D. R. OLIVEIRA
Universidade do Minho
CQPA/INIC
Av. João XXI
4700 Braga
Portugal

1. Introduction

Adhesion of microorganisms to solid surfaces is one of the prime steps in the formation of biofilms.

As regards the size of a great number of microorganisms, namely bacteria, they are quite often considered as colloidal particles (*Marshall* 1976). Consequently, microbial adhesion has been described in the literature in terms of DLVO theory, developed independently by *Derjaguin* and *Landau* (1941) and *Verwey* and *Overbeek* (1948) to explain the stability of lyophobic colloids.

According to this theory, the net force of interaction arises from the balance between *van der Waals* forces of attraction and electrostatic double-layer forces, those having commonly a repulsive effect.

However, apart from DLVO forces other types of interactions can play an important role in the adhesion process, specially hydrophobic interactions in aqueous medium and the steric hindrances in the presence of polymers.

Moreover it has to be kept in mind that those "living colloids" are capable of excreting polymeric metabolites which along with external appendages can have a strong influence in the final process of adhesion.

It is relevant to note that prior to adhesion the microorganisms have to be transported to the surface of deposition. Transport mechanisms can be very different, because adhesion takes place either in quiescent waters or in turbulent flow conditions (*Characklis* 1981a; 1981b) The transport flux of microorganisms to the surface of deposition must be directly proportional to their bulk concentration in accordance with mass transfer theories. Therefore, in very dilute suspensions transport can be the controlling step in the overall process of deposition.

In flow systems, the effect of hydrodynamic forces has also to be stressed, because their removal action can limit the extent of biofilm growth.

Some of the aspects mentioned so far will be outlined in more detail in the following survey.

2. Colloid Chemistry and Adhesion

2.1. *VAN DER WAALS* INTERACTIONS

The existence of attraction forces between molecules was proposed by *van der Waals* (1873) as a result of his studies on the deviations from the ideal-gas law. About 60 years later, with the emergence of quantum mechanics, *London* (1930) quantified this statement

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 45–58.

and *Hamaker* (1937) based on the pairwise principle, extended *London's* theory to the interaction between solid bodies.

van der Waals interactions are dependent on the geometry and on the nature (physical and chemical properties) of the interacting bodies. This last feature is included in a constant, **A**, named after *Hamaker*.

In the case of bacterial adhesion the interacting geometries are often assumed to be of the sphere/plate type and the expression for the *van der Waals* force (F_W) is:

$$F_W = \frac{AR}{6d^2} \tag{1}$$

where **R** is the radius of the spherical particle and **d** is the separation distance.

Those interactions are more commonly treated in terms of energy (V_W) rather than force, but as F = dV/dd the energy is given by:

$$V_W = -\frac{AR}{6d} \tag{2}$$

Expressions for other geometries can be found in the literature (Hiemenz; Vold 1954).

2.1.1. *Hamaker Constants*. The *Hamaker* constant can be calculated from the molecular properties of the materials involved. For the interaction of two different materials 1 and 2 in vacuum the corresponding *Hamaker* constant can be obtained in good approximation through:

$$A_{12} = \sqrt{A_{11}.A_{22}} \tag{3}$$

If those materials are immersed in a medium 3, the interaction constant may be given by:

$$A_{132} = (\sqrt{A_{11}} - \sqrt{A_{33}}) \cdot (\sqrt{A_{22}} - \sqrt{A_{33}}) \tag{4}$$

Several approaches have been used to calculate the A_{ii} constants, most of them based on the *Hamaker's* assumption of molecular additivity, which is not strictly valid for condensed media interactions. *Visser* (1972) has an excellent review on *Hamaker* constants.

Lifshitz and collaborators (*Dzyaloshinskii* 1961), avoided the additivity principle and were able to calculate the *van der Waals* interactions between macroscopic bodies using the characteristic electromagnetic spectrum absorption frequencies. The resulting equation is complex and some simplifications have been tried. *Israelachvili* (1974) assumed that the major contribution for the dispersion interaction arises from electronic excitation in the ultraviolet frequency range and he obtained the following equation:

$$A_{ii} = \frac{3}{16\sqrt{2}} \cdot \frac{(n_o^2 - 1)^2}{(n_o^2 + 1)^{1.5}} \hbar \omega_{UV} \tag{5}$$

$\mathbf{n_o}$ is the refractive index in the visible range, $\hbar$ is *Planck's* constant and ω_{UV} is the characteristic absorption frequency in the UV region.

However, there are still difficulties in obtaining the absorption spectra in the far ultraviolet for a great number of substances.To circumvent this problem, *Van Oss* and co-workers (1988) decided to calculate *Hamaker* constants for a series of liquids for which the values of *Lifshitz-van der Waals* component of the surface tension (γ^{LW}) (see 2.5) were known and for which the necessary spectroscopic data were available as well. Assuming that the equilibrium separation (d_o) between the interacting bodies is determined by the

balance between the *Born* repulsion and the *van der Waals* attraction they obtained the following expression:

$$A_{ii} = \gamma_i^{LW} \cdot 32 \pi d_o^2 \qquad (6)$$

The average value found for $\mathbf{d_o}$ is 0.136 nm, with a standard deviation of 0.007 nm (*van Oss et al.*1988).

An interesting feature of the *Hamaker* constants is the possibility of obtaining negative values for A_{132}. This happens when

$$A_{11}<A_{33}<A_{22} \qquad \text{or} \qquad A_{11}>A_{33}>A_{22}$$

Consequently, the interaction between the two different materials is weaker than the interaction between each of them and the medium in which they are immersed. So, if the above conditions are fulfilled a spontaneous separation is likely to occur on account of the dispersion forces only (*Visser* 1975).

2.2. DOUBLE-LAYER FORCES

2.2.1. *Electrostatic Double-Layer*. The majority of solid bodies acquire electrical surface charge when immersed in aqueous media. Different mechanisms can be responsible for this charge acquisition (*Hunter* 1988), but in the case of biological systems this is generally due to ion adsorption or ionization of surface groups, phenomena that are very often dependent on the pH of the medium. At normal conditions of pH, bacteria possess a net negative surface charge. However, a charge reversal may occur at low pH values on account of the presence of some charged basic (amino) groups (Plummer and James 1961).

A charged surface immersed in an aqueous medium will promote a redistribution of ions. The ions of the same sign (co-ions) will be repeled from the surface while the oppositely charged ions (counter-ions) will be attracted. This effect along with the Brownian motion gives rise to a *Poisson-Boltzmann* distribution of the ions through out the aqueous phase creating a diffuse layer which, together with the solid surface, is called the electrical double-layer (Figure 1).

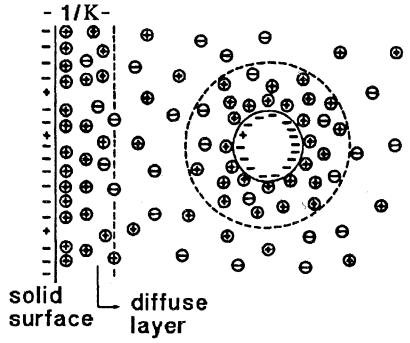


Figure 1. - Electrostatic double-layer around a flat surface and a spherical particle.

The electrostatic potential decreases from the charged surface through the diffuse layer, attaining a null value in the bulk.

When two charged bodies approximate, the interpenetration of their double-layers promotes the repulsion between them. This repulsive effect is the most common, because, as already mentioned for bacteria, the majority of solid bodies also acquire a net negative charge when in aqueous environments.

2.2.2. *Double-Layer Interactions.* The potential energy arising from the interpenetration of electrical double-layers depends on the geometry of the interacting bodies and on the electrical behaviour during the interaction. Generally, the interactions are considered to occur at constant surface potential or at constant surface charge and the most commonly used equations, for the sphere/plate type geometry, are the equation of *Hogg Healy* and *Fuerstenau* (1966) (eq.(7)) and the equation of *Wiese* and *Healy* (1970) (eq.(8)), respectively.

$$V^{\psi}_{DL_{sp/pl}} = \varepsilon\pi R \{(\psi_{o1} + \psi_{o2})^2 \ln[1+\exp(-\kappa d)] + (\psi_{o1} - \psi_{o2})^2 \ln[1-\exp(-kd)]\} \quad (7)$$

$$V^{\sigma}_{DL_{sp/pl}} = - \varepsilon\pi R \{(\psi_{o1} + \psi_{o2})^2 \ln[1-\exp(-\kappa d)] + (\psi_{o1} - \psi_{o2})^2 \ln[1+\exp(-kd)]\} \quad (8)$$

V^{ψ}_{DL} and V^{σ}_{DL} are the potential energies of interaction at constant surface potential and at constant surface charge, respectively. ψ_{o1} and ψ_{o2} are the surface potentials of the flat surface and of the spherical particle at infinite separation. ε is the electrical permittivity of the medium, **R** is the sphere radius and κ is the reciprocal double-layer thickness or Debye--Huckel parameter, given by:

$$\kappa^2 = \frac{1000\, e^2\, N_A}{\varepsilon\, K_B\, T} \Sigma\, z_i^2\, M_i \quad (9)$$

Where **e** is the electron charge, N_A is the *Avogadro's* number, K_B is the *Boltzmann* constant, **T** is the absolute temperature, z_i is the counter-ion valence and M_i is the counter--ion molarity.

The HHF and HW equations are based on the linearization of *Poisson* and *Boltzmann* equations and are only valid for surface potentials <25 mV, although they can be used without significant errors for potentials until 60 mV (*Rajagopalan* and *Kim* 1981).

The condition of constant surface potential may only be fulfilled if the surface charge is created by the adsorption of "potential determining ions", while the situation of constant surface charge arises when the charge is originated by isomorphic substitutions inside the lattice (*Rajagopalan* and *Kim* 1981).

The intermediate situations, where neither the charge nor the potential are kept constant, occur when the surfaces acquire electrical charge due to the ionization or dissociation of surface groups (*Rajagopalan* and *Kim* 1981), which seems to be the most common case for biological systems.

In such situations, *Gregory* (1975) considers that the best approximation is given by the equation of *Bell Levine* and *McCartney*. *Kar et al.* (1973) derived an expression for the interaction between a surface at constant charge and a surface at constant potential.

Frens and *Overbeek* (1972) assume that the condition of constant potential is impossible during an interaction, only the charge can be kept constant. A similar consideration is also given by *Weaver* and *Feke* (1985).

Lyklema (1980), introducing the concept of double-layer relaxation, assumed that both situations of constant potential and constant charge are extreme cases. The real situation is determined by the relaxation rate of the different double-layer regions (e.g. diffuse layer and/or *Stern* layer).

Although there is such a controversy, most of the authors utilize the HHF equation on account of its simplicity.

An important point to be noted is that it is still impossible to determine the exact values of the surface potentials. Those values are replaced in the equations by the corresponding zeta potential values (Hunter 1988).

2.3. DLVO THEORY

According to this theory, the total potential energy of interaction (V_T) is obtained summing up the energy due to *van der Waals* interactions (V_W) and the energy arising from double--layer interactions (V_{DL}):

$$V_T = V_W + V_{DL} \tag{10}$$

Conventionally, the repulsive interactions are positive and the attractive interactions are affected by a minus sign. This makes possible to speak about the "depth" of the minima of energy and the "height" of the energy barrier. Therefore, in the most common case, where only the *van der Waals* forces are attractive a possible profile for the total potential energy of interaction is given in Figure 2.

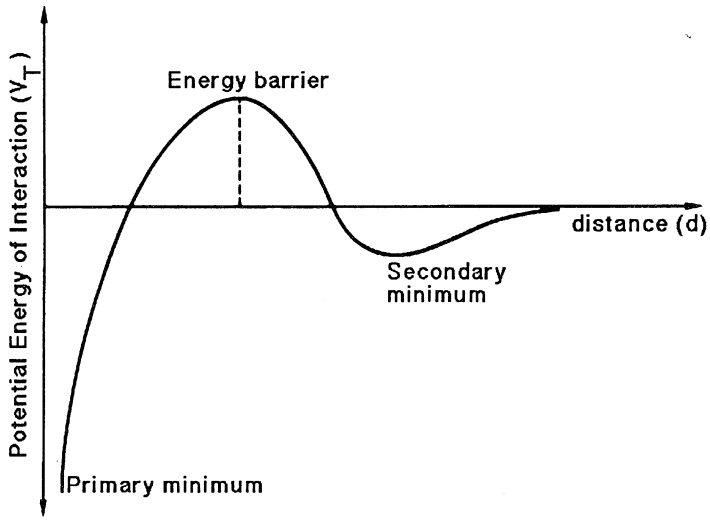


Figure 2. - Total potential energy profile according to DLVO theory.

The interacting bodies will attain the maximum stability in the primary minimum of energy.

The possibility of existing two energy minima enables to explain adhesion in terms of reversibility and irreversibility. In a situation of reversible adhesion the stabilazation occurs in the secondary minimum of energy. In these circumstances the microorganisms are still

capable of Brownian motion and can be removed by washing. In irreversible adhesion the primary minimum is attained, all Brownian motion ceases and a simple washing procedure is not enough for the removal of the deposited materials.

As can be seen from the equations concerning the energies of interaction the energy profile is influenced by several parameters. The effect of the different parameters on the energy profile was shown by *Ruckenstein* and *Kalthod* (1981). The most common example shown is the effect of the ionic strength. An increase in the ionic strength lowers the energy barrier favouring adhesion.

2.4. OTHER FORCES

There is now experimental evidence of other types of forces acting at short or intermediate distances, being attractive or repulsive, which can play an important role in the process of adhesion.

2.4.1. *Hydrophobic Interactions*. Hydrophobic interaction is generally so called when being attractive, while its repulsive counterpart is commonly referred to as "hydration pressure". These interactions are of polar nature and can have a magnitude up to two decimal orders higher than the components of DLVO theory (*van Oss et al.* 1988). *Van Oss* and collaborators (1987) considered that those forces are based on electron donor-electron acceptor (*Lewis* acid-base) interactions (section 2.1) between polar species in polar media (e.g. water) and are responsible for almost all the anomalies found in the interpretation of interfacial interactions in polar media.

Based on the works of several investigators *van Oss et al.* (1988) proposed an equation for the calculation of the free energy (ΔF^{AB}) associated with this type of forces, for the parallel flat plate conformation. Using the approach of *Derjaguin* (*Hogg et al.* 1966) it is possible to obtain the equation for the sphere/plate type geometry:

$$\Delta F^{AB}_{sp/pl} = 2\pi R \lambda \Delta F^{AB}(do) \exp^{(do-d)/\lambda} \qquad (11)$$

Where λ is the correlation lenght pertaining to water molecules, **do** is the equilibrium distance and **d** is the distance. This equation is valid for $d>\lambda$. For pure water the value of λ is around 0.2 nm, but at higher ionic strengths is related to the dimensions of the hydrated ions, having values up to 1.2 nm (*van Oss et al.*1988). ΔF^{AB} **(do)** is the free energy at the distance of equilibrium and can be calculated through the electron acceptor (γ^+) and electron donor (γ^-) parameters of the polar components (γ^{AB}) of the surface tension of the interacting bodies (*van Oss et al.* 1987).

The polar component of the free energy of interaction between materials 1 and 2 immersed in a medium 3 is expressed by (*van Oss et al.*1987):

$$\Delta F^{AB}_{123}(do) = 2\left[\sqrt{\gamma_3^+}\left(\sqrt{\gamma_1^-}+\sqrt{\gamma_2^-}-\sqrt{\gamma_3^-}\right)+\sqrt{\gamma_3^-}\left(\sqrt{\gamma_1^+}+\sqrt{\gamma_2^+}-\sqrt{\gamma_3^+}\right)-\right.$$
$$\left.-\sqrt{\gamma_1^+\cdot\gamma_2^-}-\sqrt{\gamma_1^-\cdot\gamma_2^+}\right] \qquad (12)$$

ΔF^{AB}, as expressed by equation (11), has the dimensions of energy (joule) and so it is suggested that it can be introduced , as a third term, in equation (10). This means that the DLVO theory is extended, in order to contemplate the hydrophobic interactions.

2.4.2. *Steric Forces*. This type of forces is considered to arise between polymer coated surfaces. The potential energy of interaction between two uncharged polymer coated

surfaces is complex, but essentially comprises contributions from three additive terms (*Vrij* 1976 ; *Napper* 1977 ; *Scheutjens* 1982):

i - a mixing term related to the polymer segment concentration in the interacting zone;
ii - an elastic term related to the loss of configurational entropy of the polymer chain;
iii. - an adsorption or bridging term, being important at low coverages.

The situation becomes even more complex when the surface layers are charged or are polyelectrolytes.

It is expected that those forces may be relevant in biological systems, where the existence of macromolecules (e.g. glycoproteins, polysaccharides, lipopolysaccharides, etc.) are quite common. The adsorption of these macromolecules, which may be neutral polymers or polyelectrolytes, can occur before any appreciable microbial adhesion takes place, giving rise to the so called surface conditionning. However, in the present state of the art it does not seem possible to quantify steric forces, on account of their complexity.

2.5. SURFACE FREE ENERGY APPROACH

According to *Absolom et al.*(1983), bacterial adhesion is possible if the process causes the free energy to decrease:

$$\Delta F^{ad} < 0 \qquad (13)$$

The change in the free energy of adhesion (ΔF^{ad}), when electrical charge interactions are neglected, can be determined by:

$$\Delta F^{ad} = \gamma_{sb} - \gamma_{sl} - \gamma_{bl} \qquad (14)$$

with γ_{sb} - interfacial tension substratum-liquid phase

γ_{sl} - interfacial tension substratum-liquid phase

γ_{bl} - interfacial tension bacteria-liquid phase

There are different approaches for the calculation of the interfacial tension values, because there are distinct insights on the nature of the surface tension. However, all those approaches are based on *Young's* equation (*Fowkes* 1967), that correlates the contact angle (θ), formed by a liquid drop on a solid surface with the interfacial free energies of the three contacting phases:

$$\cos\theta\ \gamma_{lv} = \gamma_{sv} - \gamma_{sl} \qquad (15)$$

Where **l** stands for liquid, **v** for vapour and **s** for solid.

Neumann et al. (1974) assume a single component surface tension and derived an "equation of state" for the calculation of γ_{sv} and γ_{sl}.

Busscher (1984) considering the existence of a polar (γ^p) and a dispersion (γ^d) component of the surface tension, proposed the following equation for the calculation of the surface tension of the solid susbstratum ($\gamma_s = \gamma_s^p + \gamma_s^d$).

$$\cos\theta = -1 + \frac{2}{\gamma_l}\left(\gamma_s^d.\gamma_l^d\right)^{1/2} + \frac{2}{\gamma_l}\left(\gamma_s^p.\gamma_l^p\right)^{1/2} - \frac{\pi e}{\gamma_l} \qquad (16)$$

πe is the "spreading pressure", defined as the difference between the free energy of the solid in the air (γ_S) and the free energy in the presence of vapour molecules (γ_{SV}) of the liquid used in contact angle measurements ($\pi e = \gamma_S - \gamma_{SV}$). Having determined γ_s^d, γ_s^p and πe, by means of a least square fitting of the data obtained using a defined series of liquids, it is possible to calculate γ_{Sl} (or generally γ_{ij}) through:

$$\gamma_{ij} = \gamma_i + \gamma_j - 2\left(\gamma_i^d.\gamma_j^d\right)^{1/2} - 2\left(\gamma_i^p.\gamma_j^p\right)^{1/2} \qquad (17)$$

More recently, *van Oss et al.* (1987) considered that the surface tension (γ) of a given substance comprises a component arising from the *Lifshitz-van der Waals* interactions (γ^{LW}) (of the *London + Keesom + Debye* varieties) and a component due to polar interactions (γ^{AB}) of the type electron acceptor-electron donor, similar to *Lewis* acid-base (AB) interactions, which include the special case of hydrogen donor-hydrogen acceptor interactions.

The surface tension can then be expressed by:

$$\gamma = \gamma^{LW} + \gamma^{AB} \qquad (18)$$

with $$\gamma^{AB} = 2\sqrt{\gamma^+.\gamma^-} \qquad (19)$$

Where γ^+ and γ^- are the electron acceptor and the electron donor parameters, respectively.

γ^{LW}, γ^+ and γ^- can be determined by contact angle measurements (*van Oss et al.* 1988).

In certain circumstances, the thermodynamic approach has been used with some success to explain bacterial adhesion (*Bellon-Fontaine et al.* 1990).

2.5.1. *Substratum Wettability*. One of the liquids that is commonly used in contact angle measurements is water. The data obtained with water reflect a special feature of solid surfaces, that is named wettability. The degree of wettability of a surface is variable, it can be low or high and, in accordance, surfaces can be divided into hydrophobic or hydrophilic.

Baier (1973), based on experience with some biological systems, predicted that minimal bacterial adhesion in aquatic habitats should occur on substrata within a critical surface tension range of 20 to 30 mN/m. Those values pertain to low energy (or hydrophobic) surfaces and so, maximal adhesion should be expected on high energy (hydrophilic) substrata. However, *Fletcher* and co-workers (1979 and 1984) observed a different trend in some situations and in others they could not find any generic pattern of attachment according to surface wettability. They have expressed the substrata hydrophobicity in terms of work of adhesion (W_A), obtained via the *Young-Dupre* equation:

$$W_A = \gamma_{lv}\left(1 + \cos\theta_E\right) + \pi e \qquad (20)$$

Where θ_E is the equilibrium contact angle for water. For adhesion to occur between hydrated species water must be displaced as the two surfaces move closer. W_A represents the work that is necessary to displace the water molecules.

With such conflicting results the concept of wettability, alone, does not seem to be a good approach to predict bacterial adhesion.

It must be stressed that although the surface energy and the colloid-chemical approaches are often considered as separate entities, there are strong connections between them, as was shown by *Pethica* (1980).

3. Adhesion in Flowing Systems

Most of the studies on biofilm formation deal with systems exposed to little or no fluid motion. However, there are situations, like in industrial processes where there is significant fluid flow.

The fluid velocity seems to have two opposite effects on biofilm formation. An increase in fluid velocity increases the shear stress exerted on the deposited microorganisms which can promote their detachment. On the other hand, it makes possible an increase in the mass transfer rate of nutrients to the surface, which may be responsible for a higher biofilm growth. It has been reported that biofilms formed under higher velocity conditions may adhere more firmly to surfaces than the ones developed at lower velocities (*How* et al. 1982; *Dudddrige et al.*1982; *Vieira et al.*(a)), problably this is due to a stronger choesion promoted by the higher shear stresses and to a higher production of extracellular polymers (in a more adverse environment) making the deposit more "stickier".

Some authors assume that the removal effects of hydrodynamic forces on deposited materials can only be explained if a short range repulsive force is considered in conjunction with DLVO theory, in order to make possible a finite primary minimum in the energy profile (Figure 3) (*Ruckenstein* and *Kalthod* 1981; *Ruckenstein* 1978; *Kallay et al.* 1986). Otherwise, only a force of infinite magnitude could promote the re-entrainment of deposited particles. A similar reasoning was suggested by *Hamaker* (1937), when studying the repeptization (defloculation) of colloidal particles.

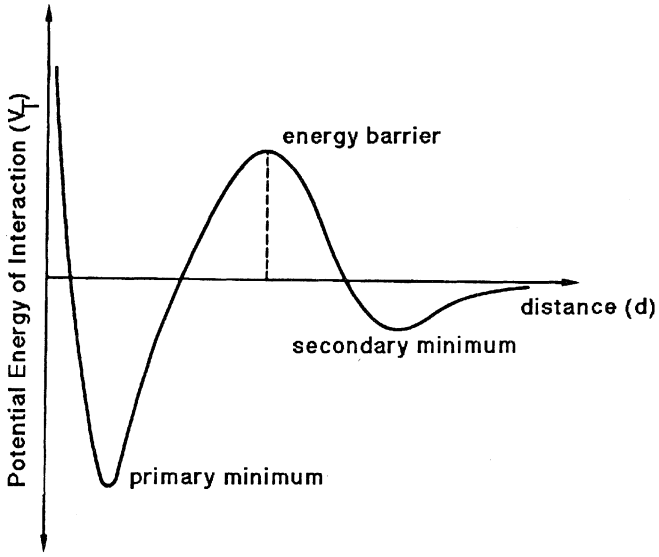


Figure 3. - Total potential energy profile considering the effect of *Born* repulsion.

The short range force considered so far, is the *Born* repulsion. For the sphere/plate conformation, when R>>d, it can be expressed by:

$$V_{BR_{sp/pl}} = \frac{A\, d_o^6\, R}{168\, d^7} \qquad (21)$$

However, *Frens* and *Overbeek* (1972) consider that it is not possible for the superficial atomic orbitals of two coagulated particles to interpenetrate to give rise to a *Born* repulsion. Although they have considered that it is higly improbable that a Brownian collision could bring two particles closer than twice the distance between the particle surface and the outer *Helmhotz* plane of the electrical double-layer.

4. Microbiological Aspects

Beyond all the forces mentioned above, that can be involved in the adhesion process, an additional difficulty arises in studying microbial adhesion, because living systems are being concerned. This condition of living organisms is expressed by the abbility to reproduce, grow and produce extracellular polymers and external appendages and, in some cases, to move independently.

4.1. EXTERNAL APPENDAGES

The external appendages are present in a great number of bacteria and are commonly divided into three different types:

. flagella - very fine threads with an helical structure protuding from the cytoplasm through the cell wall, responsible for bacteria motility;

. pili or fimbriae - very fine threads, shorter than flagella. One cell may have several of this structures appearing like tufts;

. stalks and prostechae - filiform or blunt extensions of the cell wall and membrane that can occur in one or more sites on the cell surface, respectively.

The latter two types of appendages are not involved in motility and are commonly considered as specialised attachment structures (Kent and Duddridge 1981; Paerl 1975; Handley et al.1991). In many prostechae and specially in stalks there is a disc in the end, called the "hold-fast", which has been referred to in the literature as the preferential attachment structure of diatoms (Characklis and Cooksey 1983).

The attachment through these fillamentous structures can be interpreted as a means to favour adhesion, because it reduces the effective radius of interaction and concomitantly lowers the energy barrier, as predicted by DLVO theory. However, it must be noted that, although present, they are not always involved in the adhesion process (Characklis and Cooksey 1983).

4.2. EXTRACELLULAR POLYMERS

The second major means of microbial attachment is thought to be the extracellular polymers. Extending lengths of polymers attached to cell surfaces can interact with vacant bonding sites on the surface - polymer bridging - and as a result the cell is hold near the surface. Possible mechanisms for polymer bridging have been suggested (*Characklis* and *Cooksey* 1983; *Kent* and *Duddrige* 1982) but they are not yet fully understood.

4.3. ION BONDING

Inorganic ions may also affect microbial attachment to surfaces. In the case of Ca^{2+} and Mg^{2+}, claims have been made that they are fundamental for an efficient adhesion of aquatic bacteria (*Marshall* and *Stout* 1971; *Fletcher* and *Floodgate* 1973). Different mechanisms have been proposed for the role of divalent cations in the process of bacterial adhesion, namely the formation of a cation bridge between the negatively charged bacteria and the substratum (*Fletcher* and *Floodgate* 1973) and the precipitation of polymer, mediated by the cation, between the cell and the substratum (*Rutter* 1980). More recently, *Van Oss et al.* (1987) proposed that Ca^{2+} can depress the monopolar electron-donor parameter of the surface tension of the interacting species, depressing their capacity for mutual repulsion and their degree of hydration, resulting in a decrease of the hydration pressure.

The effect of other cations is complex, because some favour adhesion and others do not (*Duddrige et al.* 1981; *Daniels* 1972), possibly by affecting the adhesive properties of the extracellular polymers. However, these results cannot be interpreted straighforward, since the ions may affect cell metabolism and viability (*Vieira* et al.(b)).

5. Conclusions

The explanation of microbial adhesion have been tried in terms of colloid chemistry theories, specially DLVO theory, which only takes into account long range forces. However, there is now the possibility of quantifying other types of forces, namely hydrophobic interactions, and they must be associated to DLVO forces in order to have a better defined energy profile. Nevertheless, even doing this, it is not possible to have a fully characterised picture of the adhesion process of microorganisms They have to be regarded as "living colloids" with their adaptable and varied nature, being capable of very special types of interactions. Therefore, much more has to be known!

Nomenclature

ΔF^{ad} - change of free energy of adhesion (J/m^2)
K_B - Boltzmann constant (J/K)
e - electrical charge of electron (C)
$\Delta F^{AB}_{sp/pl}$ - change of free energy associated to hydrophobic interactions between a sphere and a flat plate (J)
ΔF^{AB}_{123} - polar component (acid-base) of the surface free energy between bodies 1 and 2 in medium 3 (J/m^2)
ΔF^{ad} - change of free energy of adhesion (J/m^2)
K_B - Boltzmann constant (J/K)
M_i - counter-ion molarity (mol/dm^3)
N_A - Avogadro's number
n_o - refractive index in the visible range
R - radius of particle (m)
T - absolute temperature (K)
V_{BR} - potential energy associated with Born repulsion (J)
V_T - total potential energy of interaction (J)
V_W - potential energy associated to van der Waals interactions (J)
V_{DL} - potential energy due to double-layer forces (J)

V_{DL}^{ψ} - potential energy due to double-layer forces at constant surface potential (J)

V_{DL}^{σ} - potential energy due to double-layer forces at constant surface charge (J)

W_A - work of adhesion (J/m^2)

z_i - valence of ion **i**

ε - electrical permittivity (F/m)

γ_{ij} - interfacial free energy between bodies **i** an **j** (J/m^2)

γ_{bl} - interfacial free energy between bacteria and a liquid phase (J/m^2)

γ_{sb} - interfacial free energy between a solid surface and bacteria (J/m^2)

γ_{sl} - interfacial free energy between a solid surface and a liquid phase (J/m^2)

γ^p - polar component of the surface tension (J/m^2)

γ^d - dispersion component of the surface tension (J/m^2)

γ^{AB} - acid-base component of the surface tension (J/m^2)

γ^{LW} - Lifshitz-van der Waals component of the surface tension (J/m^2)

γ^+ - electron acceptor parameter of the γ^{AB} surface tension component

γ^- - electron donor parameter of the γ^{AB} surface tension component

h - Planck's constant (J.s)

λ - correlation length of water molecules (m)

ψ_{oi} - electrical potential of surface i (V)

ω_{UV} - characteristic absorption frequency in the UV range (rad/s)

π_e - spreading pressure (J/m^2)

θ - contact angle (degree)

References

Absolom D.R., Lamberti F.V., Policova Z., Zingg W., van OSS C.J. and Neumann A.W., (1983) Appl. Environ. Microbiol. **46**, 90-97.

Baier R.E., (1973) "Influence of the Initial Surface Condition of Materials on Bioadhesion", Proc. 3rd International Congress on Marine Corrosion Fouling, in R.F. Acken, B.F. Brown, J.R. DePalma and W.P. Iverson (eds.), Northwestern University Press, Evanston, III, pp 633-639.

Bellon-Fontaine M.N., Mozes N., van der Mei H.C., Sjollema J., Cerf O., Rouxhet P.G. and Busscher H.J., (1990) Cell Biophysics **17**, 93.

Busscher H.J., (1984), Thesis, University of Groningen.

Characklis W.G., (1981 a) Biotech Bioeng. **23,** 1923-1960.

Characklis W.G., (1981b) "Microbial Fouling: a Process Analysis" in Fouling of Heat Transfer Equipment, E.F.C. Somerscales and J.G. Knudsen (eds.), Hemisphere, Washington DC, pp 251-291.

Characklis W.G. and Cooksey K.E., (1983) Adv. Appl. Microbiol. **29**, 93-138.

Daniels S.L., (1972) Dev. Ind. Microbiol. **13,** 211-253.

Derjaguin B.V. and Landau L., (1941) Acta Physicochim. URSS **14**, 633-662.

Dexter S.C., (1979) J.Coll. Int. Sci. **70**, 346-354.

Dexter S.C., Sullivan J.D. Jr., Williams J. III and Watson S.W., (1975) App. Microbiol. **30**, 298-308.

Duddrige J.E., Kent C.A. and Laws J.F., (1981) "Bacterial Adhesion to Metallic Surfaces", I Corrosion Sci. Tech./I.Chem. E. Conference on Prevention of Fouling in Industrial Plant Nottingham University.

Duddrige J.E., Kent C.A. and Laws J.F., (1982) Biotech. Bioeng. **24**, 153-164.

Dzyaloshinskii I.E., Lifshitz E.M., Pitaevskii L.P.,(1961), Adv. Phys. **10**, 165.

Fletcher M. and Floodgate G.D., (1973) J. Gen. Microbiol. **74**(2), 325-334.

Fletcher M. and Loeb G.I.,(1979) Appl. Environ. Microbiol. **37**, 67-72.

Fletcher M., Bright J.J. and Pringle J.H.,(1983) "Attachment of Aquatic Bacteria to Solid Surfaces", in Fouling of Heat Exchanger Surfaces, R.W. Bryers, Cole S.S. (eds.), Engineering Foundation, NY, 33-49.

Fowkes F.M., (1967) "Contact Angle - Wettability and Adhesion" , ch 6, American Chemical Society (ed.), Washington DC.

Frens G., Overbeek J. Th. G., (1972), J. Coll. Int. Sci. **38**, 376-387.

Gregory J., (1975) J. Coll. Int. Sci. **51**, 44-51.

Hamaker H.C., (1937) Physica (Utrecht) **4**, 1058.

Handley P.S., Hesketh L.M. and Moumena R.A., (1991) Biofouling **4**, 105-111.

Hiemenz P.C., "Principles of Colloid and Surface Chemistry", Marcel Dekker, N.Y.

Hogg R., Healy T.W. and Fuerstenau D.W., (1966) Trans.Faraday Soc.**62**,1638-1651.

How I., Duddrige J.E., Kent C.A. and Pritchard A.M., (1982) "the Effects of Shear Stress on BiofoulingDeposit Formation and Removal", H.T.F.S. Research Symposium, Warwick University, 14-17.

Hunter R.J., (1988) "Zeta potential in Colloid Science", Academic Press, London

Israelachvili J.B.Q., (1974) Rev. Biophys. **6**, 341.

Kallay N., Biskup B., Tomic M. and Matijevic E.,(1986) J.Coll. Int. Sci. **114**, 357-362.

Kar G., Chander S. and Mika T.S., (1973) J. Coll. Int. Sci. **44**, 347-355.

Kent C.A. and Duddrige J.E., (1981) "Microbial fouling of Heat Transfer Surfaces in Cooling Water Systems". AERE Report R 10065, United Kingdom Atomic Energy Authority.

London F., (1930) Z. Phys. **63**, 245.

Lyklema J., (1980) Pure & App. Chem. **52**, 1221-1227.

Marshall K.C., (1976) "Interfaces in Microbial Ecology", Harvard University Press, Cambridge, Massachussets.

Marshall K.C. and Stout R., (1971) J. Gen. Microbiol. **68**, 337-348.

Napper D.H., (1977) J. Cool. Int. Sci. **58**, 390-407.

Neumann A.W., Good R.J., Hope C.J. and Sejpal M., (1974) J. Coll. Int. Sci. **49**, 291--304.

Paerl H.W., (1975), Microbiol. Ecol. **2**, 73-83.

Pethica B.A., (1980) "microbial and Cell Adhesion: Microbial Adhesion to Surfaces", in R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter and B. Vincent (eds.), Ellis Horwood, Chichester 19-45.

Plummer D.T., James A.M., (1961) Biochim Biophys Acta **53** 453-460.

Rajagopalan R. and Kim J.S., (1981) J. Coll. Int. Sci. **83**, 428-448.

Ruckenstein E. (1978) J. Coll. Int. Sci. **66**, 531-543.

Ruckenstein E. and Kalthod D.G.(1981) "Role of Hydrodynamics and Physical Interactions in the Adsorption and Desorption of Hydrosols or Globular Proteins" in Fundamentals and Applications of Surface Phenomena Associated with Fouling and Cleaning in Food Processing, B. Hallstrom, D.B. Lund, Ch. Tragardh (eds.), Tylosand, Sweden, 115-167.

Rutter P.R., (1980) "Cell Adhesion and motillity", in A.S.G. Curtis and J.D. Pitts (eds.), Cambridge University Press, London, pp 103-135.

Scheutjens J.M.H.N. and Fleer G.J., (1982) Adv. Coll. Int. Sci. **16**, 361-380.

van der Waals J.D., (1873), Thesis, Leiden.

van Oss C.J., Chaudhury M.K., Good R.J., (1987) Adv. Coll. Int.Sci. **28**, 35-64.

van Oss C.J., Chaudhury M.K. and Good R.J., (1988), Chem Reviews **88**, 927-941.

Verwey E.J.W. and Overbeek J.T.G., (1948) "Theory of the Stability of Lyophobic Colloids", Elsevier Amsterdam.

Vieira M.J., Oliveira R., Melo L., Pinheiro M. and van der Mei H.C., (1992a) Biocoll. Biosurf., in press

Vieira M.J., Oliveira R., Melo L., Pinheiro M.M. and Martins V., (1992b) submitted to Coll. and Surfaces.

Visser J., (1972) Adv. Coll. Int. Sci. **3**, 331-363

Visser J., (1973) Thesis, Council for National Academic Awards, London.

Visser J., (1975) "Adhesion of Colloidal Particles", in E. Matijevic (ed.), Surface and Colloid Science, John Wiley & Sons, Inc., NY.

Vold M.J., (1954) J. Coll. Sci. **9**, 451-459.

Vrij A., (1976) Pure Appl. Chem. **48**, 471-483.

Weaver D.W., Feke D.L.(1985) J. Coll. Int. Sci. **103**, 267-283

Wiese G.R.and Healy T.W., (1970) Trans. Faraday Soc. **66**, 490

THE ROLE OF ADSORBED LAYERS IN BACTERIAL ADHESION.

A.H.L.CHAMBERLAIN
School of Biological Sciences
University of Surrey
Guildford GU2 5XH, United Kingdom

1. Introduction

There is now an enormous literature to support the concept that bacteria in aqueous environments are predominantly associated with surfaces (Costerton et al.,1987; Characklis and Marshall,1990). The development of a biofilm is perceived as a multi-stage process, of which the initial step is adsorption of material to the newly immersed surface. The nature of the adsorbate(s) depend upon the surface itself but in virtually every system investigated the establishment of an adsorbed layer can be demonstrated. The rate at which this occurs is in part controlled by the bulk concentration of the adsorbates, their relative affinities for the surface and the hydrodynamic environment.

This adsorbed film has frequently been termed a "conditioning" layer and there has been some discussion as to whether or not it is a pre-requisite for subsequent bacterial attachment. The problem is rather difficult to resolve as it is unlikely that any surface is adsorbate-free before organism attachment occurs, as adsorption begins immediately on immersion. Indeed if the air-water interface is passed through slowly, then the new substratum may pick up a Langmuir-Blodgett type film instantaneously, depending upon its wettability, i.e. if hydrophobic it will gain a layer on the down trip, if hydrophilic on the up (Loeb, 1965). The formation and nature of the adsorbed layer has been investigated in many different subject areas and the development of an integrated model may now be attempted, drawing upon experience from the medical, food, environmental and "pure" surface chemistry disciplines.

2. The Nature of the Adsorbed Layer.

This depends very much upon the environment to which the surface is exposed, but the major components are likely to be organic. Arrival at the surface from the bulk solvent will be mediated by mass transfer (advection) or molecular diffusion; which of these processes is most

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 59–67.

important depends upon the degree of turbulence. The onset of adsorbed layer formation is extremely rapid compared with the arrival of bacteria (Table 1).

TABLE 1.Elemental composition of surface organics on stainless steel after immersion in the sea.

Duration of immersion	Relative atomic percentage				
(min)	C	O	N	Cr	Fe
0	48.18	44.01	0.78	2.35	4.48
10	49.78	43.17	0.89	1.84	3.94
30	53.97	34.10	1.02	1.13	2.62
60	51.05	36.09	0.79	2.03	3.31
120	49.71	36.16	1.66	2.03	3.44
240	61.82	28.16	3.12	0.72	1.20
340	58.13	26.67	2.35	0.59	0.82

Modified from :-
Barrett, S.J. (1989). Ph.D. thesis, University of Surrey

The adsorbed layers comprise organic molecules, particularly macromolecules, but they may also include metallic hydroxides or hydrated oxides and very fine clay mineral particles (Fig.1).

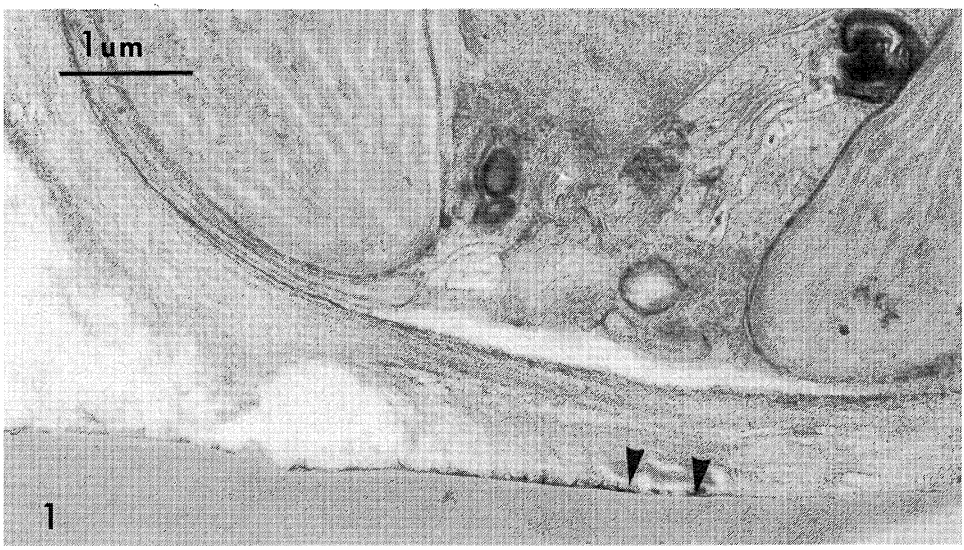


Figure 1. Adsorbed organic layer with mineral particles (arrow) below an adherent brown algal filament with a thick polysaccharide wall.

Macromolecules bind strongly to many surfaces as they frequently bear multiple attachment points, either functional groups or segments of more hydrophobic nature. Based purely on size, low molecular weight organics may frequently diffuse more rapidly to the surface and adsorb, but they are subsequently displaced by the multipoint contacting macromolecules. Once attached, these molecules are very unlikely to be easily displaced, and as indicated by Robb (1984) they are attached virtually irreversibly. Certainly, adsorption isotherms of marine organic matter adsorbed to a model substratum showed no losses on transfer to organic-free seawater (Edwards,1983).

In many medical and food processing systems, these adsorbant macromolecules are protein or glycoprotein in nature eg. the binding of casein and other milk proteins to stainless steel, or blood proteins to prostheses such as Hickman lines. In freshwater and terrestrial environments, the adsorbing species include rather more heterogeneous materials such as humic compounds and complex polysaccharides. Freshwater humic materials are primarily derived from lignin degradation products and contain large amounts of carboxylic and phenolic residues which make them very reactive. They bind metals avidly and also interact very strongly with many clay minerals, both in soils and freshwater systems. Marine humic compounds are rather different in nature and comprise complex condensation molecules with polysaccharide, peptide and lipid moeities and are primarily aliphatic (Chamberlain and van Woerkom, 1986). Terrestrial/freshwater humic compounds are usually strongly aromatic, as lignin comprises a mixture of phenylpropane derivatives.

Examination of the energy dispersive X-ray spectrum of marine organics concentrated by ultrafiltration and freeze dried, showed that they contained considerable levels of bound iron and copper. Samples produced by chloroform: water partitioning also contained quantities of very fine clay mineral particles, which showed a range of types including both expanding lattice forms and kaolinite. These minerals, particularly of such tiny dimensions, frequently only 0.1 um or less, constitute an enormous surface area for organic matter and cation interactions, and play a distinct role in overcoming the effects of pre-adsorbed organic layers. These minerals are found in the earliest phases of adsorbed layer formation and are also often associated with bacterial surfaces in natural soil and water environments.

3. Demonstration of adsorbed layers

Kristoffersen et al. (1982) using ellipsometric and surface potential measurements showed an instantaneous change in these two parameters when metals were immersed in seawater. The surface potentials stabilised after 30 minutes but ellipsometric measurements did not, and thickness continued to increase.

A comparison between the data of Kristoffersen et al. (1982) and the earlier work of Neihof and Loeb (1974, 1976) shows that within a few minutes the organic layer has achieved its typical values for wettability and surface charge, although further material continues to adsorb, yielding thicker films up to 0.08 um in 10h (Neihof and Loeb, 1976).

Barrett (1989), using X-ray photoelectron spectroscopy, showed that stainless steel pretreated for 90 hours with 0.5mg C l^{-1} of ultrafiltration extracted seawater organics, acquired an organic film.It took two days of immersion in the sea to achieve the comparable C, O, N ratios and the examination of the mean number of bacteria per mm^2 of surface using epifluorescence showed that no bacteria had attached after 10 minutes, 16.7 +/- 5.7 at 30 minutes and 107.5 +/- 10.3

after 1 hour.

Data derived from Edwards (1983) also shows very rapid adsorption of marine organics onto a prepared silver iodide substratum with 87.5 ug glucose equivalents g^{-1} after 3 hours, which increased to 93.0 after 18 hours and finally reached equilibrium at 113.5 after 90 hours.

All of these results suggest that the bulk of adsorbed film is present after 3 hours immersion in seawater. Recently, Cornelius et al. (1992) using a "real-time" adsorption system was able to show that the rate of adsorption of protein was controlled by the rate of supply and that the ^{125} I-fibrinogen was largely adsorbed within 40 minutes, reaching equilibrium at 2 hours. However, the loading levels were much higher than the seawater example.

Inevitably, this arrival at an equilibrium value gives some indication of a point of surface saturation - for that system. The data given in Table 2 drawn from Barrett (1989) show that after approximately 5 1/2 hours chromium and iron signals from a stainless steel surface are attenuated by over 80% and 75% respectively. This means that coverage is unlikely to be complete.

TABLE 2. Relative atomic percentages of C, O and N on clean and pretreated stainless steel after immersion in the sea.

Treatment	Immersion (Days)	Relative Atomic Percentage		
		Carbon	Oxygen	Nitrogen
Clean	0	53.17	45.94	0.89
	1	62.80	28.60	8.60
	2	64.52	24.23	11.25
	3	65.67	23.85	10.47
Pretreated	0	65.17	25.10	10.64
	1	64.17	25.17	10.46
	2	63.54	24.90	11.56
	3	66.17	22.98	10.86

"Clean" immersed in sterile, Organex-Milli Q water, $OmgCarbonl^{-1}$, and "Pretreated" in sterile sea water containing $0.5mg\ Carbonl^{-1}$.
Barrett, S.J.(1989) Ph.D. thesis, University of Surrey.

Loeb (1965) compared the refractive indices of a transferred protein film from a Langmuir-Blodgett device with an adsorbed film of the same material, 1.5-1.6 versus 1.2-1.4 respectively, and postulated the presence of water-filled interstices between the adsorbed globular proteins. Schrader (1982) suggested that on certain types of surface dissolved organics from seawater bound as "islands" with spaces between, and that bacteria then attached to these islands. On the contrary, it may be that bacteria attach in the spaces, leaving adhesion to be determined by the "original" substratum properties. This could account for the phenomenon of attachment correlating with properties of the original surface (eg. O'Neill and Wilcox, 1971; Dempsey, 1981; Berk et al., 1982). The alternative argument is that bacteria do bind to the organics, but that the chemistry of the substratum imposes itself upon the adsorbing organics, altering their orientation or functional group arrangement.

4. Effects of the adsorbed layer on bacterial adhesion.

The roles of adsorbed layers in microbial adhesion fall into several categories:

Modifying physico-chemical properties of the substratum.

Action as a concentrated nutrient source.

Suppression of release of toxic metal ions.

Adsorption and detoxification of dissolved inhibitory substances.

Supply of required metal trace elements.

Action as a triggerable sloughing mechanism.

Suppression of inhibitory surface polymer effects.

4.1.SURFACE PROPERTIES

The key surface physico-chemical properties which may undergo alteration are surface charge and wettability.

4.1.1 *Surface charge.* This is usually measured as microelectrophoretic mobility and data drawn from Chamberlain and Johal (1988) and Johal (1988)(Table 3) shows clearly the effect of adsorption of organic components from meat juices onto stainless steel, polyethylene and polypropylene. The tendency is to reduce the strongly negative charge of the mixed oxides on the alloy, whilst the effect on the plastics is more variable but results in imparting a charge to the plastics surfaces .

TABLE 3. Microelectrophoretic mobilities of clean and meat liquor treated processing surfaces.

Treatment liquor	Stainless steel	Polypropylene	Polyethylene
Clean	-4.17±0.03	-1.12±0.08	-0.78±0.09
Bacon	-1.96±0.01	-1.61±0.11	-0.39±0.07
Pork	-0.48±0.03	+1.09±0.03	-0.98±0.01
Beef	-0.44±0.08	+0.35±0.04	-0.27±0.01

Modified from Johal (1988). All values $x10^{-8}ms^{-1}V^{-1}$.

These results are analagous to those of Neihof and Loeb (1972a), who showed that a range of materials with widely differing surface charge values all shifted to very similar moderately negative charges on exposure to natural seawater. The values obtained were similar to those

determined on natural seawater particulates. The alteration of surface charge could be prevented by applying a U.V. photooxidation treatment to the seawater before immersion of the surfaces. In this case, with all the organics destroyed, the substrata retained their original surface charge values.

In later reports (Loeb and Neihof, 1975, 1977), examination of organic film formation on platinum surfaces in seawater showed that the change in microelectrophoretic mobility was effectively completed within the hour, although ellipsometry indicated film thickening taking place up to 5 hours and even a very reduced increase up to 20 hours.Similar results have been obtained also by Hunter (1980) examining natural estuarine particulates.

4.1.2. *Wettability*. The nature of a surface, whether hydrophilic or hydrophobic, is also effected by the adsorption of organic materials. Again the tendency is to draw the contact angle values on a wide range of substrates towards a rather narrow bracket of hydrophilic values. This can have an effect on the numbers of organisms attaching and its strength as shown by changes in the shear forces required to remove them.

4.2. NUTRIENT SOURCE.

The earliest work on the role of surfaces in the low nutrient environment by Zobell and Anderson (1936) concluded that the concentration of organics at the surface was responsible for the enhanced activity. This has been restated and demonstrated by a number of techniques of which X-ray photoelectron spectroscopy is perhaps the most convincing. Garner(1987) and Barrett(1989) were able to demonstrate the progressive accretion of organic material onto a range of substrata and to show by means of a statistical deconvolution program that the nature of the adsorbates changed with time, becoming more oxidised. This result correlates well with the observations of Baier (1973), using I.R. techniques.

4.3 TOXIC SURFACES

Most metals show at least a small degree of general corrosion and in a few cases the cations produced are toxic to microorganisms. This is particularly true of copper which develops a surface layer of copper I oxide which is quite stable below 60° C. However, small quantities of toxic copper II ions may still be released and frequently are quickly bound to adjacent organic matter. It has been shown by Campbell (1954) that surface waters contain an organic component which is believed to adsorb and prevent corrosion occurring. This effectively protects non-tolerant organisms.

4.4 DETOXIFICATION

It has been suggested that an adsorptive surface could just as likely remove toxic or inhibitory metabolic products and render them safe as adsorb nutrients. This could then lead to enhanced activity at the surface.

4.5 TRACE ELEMENTS

The binding of trace metal ions to either organic or inorganic constituents at a surface may

create a resource for organisms which can acquire them by direct uptake of the complex or release of the metal via metabolic secretions.

4.6 SLOUGHING

The adsorbed layer is often the linker between the sustratum and the organism or its attachment molecules. A shift in pH due to acid secretion will tend to enhance binding of marine organics but if the shift is in the alkaline direction then based on the isotherms of Edwards (1983) it is possible that release could occur. There are hints that other environmental changes, like sulphide generation, may also lead to sloughing.

4.7 SURFACE MODIFICATION

Finally, the adsorption of materials from the milieu may moderate the repulsion or steric interaction properties of an already established polymer as discussed in the following section.

5. Interactions of adsorbed layers and bacteria

The reason for the prevention of bacterial adhesion to surfaces carrying a well developed adsorbed organic layer appears to be either a steric interaction or a polymer hydration phenomenon. Normally an organism will tend to adsorb irreversibly to minimise the free energy of the system. However, charge repulsion or steric exclusion may prevent close approach, and even when this has been achieved ordered water structure associated very closely with the surface could still be a barrier. Hydrophilic surfaces may be stabilised by ordering of surface-associated water molecules which would be disrupted by the close approach of a bacterial cell. This releases the ordered water molecules into the bulk phase, increasing their free energy. Now hydrophobic surfaces should attract strongly because water molecules moving away from the surface into the bulk will decrease their free energy as their level of hydrogen bonding will increase (Rutter and Vincent,1980). Most bacteria are hydrophilic so an approach to a hydrophilic surface would be thermodynamically unfavourable due to the increasing free energy of the displaced water. Alternatively, the approach of a hydrophilic cell to a hydrophobic surface would yield a stronger interaction. This probably accounts for the spontaneous firm adhesion of some marine bacteria to polystyrene noted by Fletcher (1980). Hence the fact that most conditioning layers are hydrophilic will lead to poorer adhesion. This may be one reason why starving cells tend to become more hydrophobic (Kjelleberg,1984) as it would assist in adhesion to a surface where nutrients may be anticipated to have concentrated. However, as stated by Fletcher (this volume), the amount of adsorbed material at a surface is limited so the "rich harvest" will be rather short-lived.

The approach of an organism to a surface will also be controlled by the positive or negative interactions between the cell surface polymers and any adsorbed polymers at the substratum surface. If poymer quantities are low, particularly at the substratum, then there is ample opportunity for polymer bridging to take place as polymers usually have high adsorption affinities due to their multisegmental nature. Polymer interactions may be a barrier to attachment if both surfaces are well covered and the polymer loops well solvated,as this leads to steric hindrance and separation. It is at this point that adsorbed components other than

organic components come into their own. Thus, Chamberlain and van Woerkom, (1986) showed in laboratory experiments, that at very high surface loadings of organics, bacterial adhesion could be almost curtailed. When these specimens were subsequently immersed in the sea, changes to the adsorbed layer led ultimately to colonisation after some two-three weeks immersion. This loss of steric hindrance appeared to be due to the adsorption of clay mineral particles, which effectively swamped the polymer film and led to a resumption of normal colonisation. Thus, although laboratory-based work, often carried out with pure cultures, is extremely useful in understanding and predicting the behaviour of bacteria and other microorganisms on surfaces with adsorbed organic layers, it is essential to realise that these layers will be much more complex in natural or industrial systems. They can also be quickly occluded by other materials.

6. References

Baier, R.E. (1973) 'Influence of the initial surface conditions of materials on bioadhesion', Proceedings of the 3rd International Congress on Marine Corrosion and Fouling, Evanston, pp. 633-

Barrett, S.J. (1989) Marine fouling processes upon stainless steel and elastomeric surfaces, Ph.D. thesis, University of Surrey, U.K.

Berk, S.G., Mitchell, R., Bobbie, R.J., Nickels, J.S. and White, D.C. (1982) 'Microfouling on metal surfaces exposed to seawater', International Biodeterioration Bulletin, 17, 29- 39.

Campbell, H.S. (1954) 'A natural inhibitor of pitting corrosion of copper in tap-waters', J. Appl. Chem., 4, 633-647.

Chamberlain, A.H.L. and van Woerkom, R. (1986) 'The effects of adsorbed marine organics on microbial fouling', Proceedings of the 6th International Biodeterioration Symposium, Washington D.C., pp. 556-561.

Chamberlain, A.H.L. and Johal, S. (1988) ' Biofilms on meat processing surfaces', Proceedings of the 7th International Biodeterioration Symposium, Cambridge, pp. 57-61.

Characklis, W.G. and Marshall, K.C. (eds.)(1990) Biofilms, John Wiley and Sons Inc., New York..

Cornelius, R.M., Wojciechowski, P.W. and Brash, J.L. (1992) 'Measurement of protein adsorption kinetics by an in situ "real-time" solution technique', J. Coll. and Interface Sci. 150, 121-133.

Costerton, J.W., Cheng, K.-J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. (1987) 'Bacterial biofilms in nature and disease', Ann. Rev. Microbiol., 41, 435-464.

Dempsey, M.J. (1981) 'Colonisation of antifouling paints by marine bacteria', Botanica Marina, 24, 185-191.

Edwards, R. (1982) Marine dissolved organic matter: isolation, adsorption and role in primary fouling, Ph.D. thesis, University of Surrey, U.K.

Fletcher, M. (1980) 'The question of passive versus active attachment mechanisms in non-specific bacterial adhesion', in R.C.W.Berkeley, J.M.Lynch, J.Melling, P.R.Rutter and B.Vincent, Microbial Adhesion to Surfaces, Ellis Horwood, Chichester, pp.195-210.

Garner, B.J. (1987) Biofouling and corrosion studies of a copper-nickel alloy, Ph.D. thesis, University of Surrey, U.K.

Hunter, K.A. (1980) 'Microelectrophoresis properties of natural surface-active organic matter in coastal seawater', Limnol. and Oceanog.,25, 807-822.

Johal, S. (1988) 'Bacterial adhesion to processing surfaces in the meat industry', Ph.D. thesis, University of Surrey, U.K.

Kjelleberg, S. (1984) 'Adhesion to inanimate surfaces', in K.C.Marshall (ed.), Microbial Adhesion and Aggregation, Springer-Verlag, Berlin, pp. 51-70.

Kristoffersen, A., Rolla, G., Skjorland, K., Glantz, P.O. and Ivarsson, B. (1982) 'Evidence for the formation of organic films on metal surfaces in seawater', J. Coll. and Interface Sci. 86,196-203.

Loeb, G.I. (1965) 'Surface chemistry of proteins and polypeptides', U.S. Naval Research Laboratory report No.6318, pp.1-75.

Loeb, G.I. and Neihof, R.A. (1975) 'Marine conditioning films', in R.E. Baier (ed.) Applied Chemistry at Protein Interfaces, American Chemical Society, pp. 319-335.

Loeb, G.I. and Neihof, R.A. (1977) 'Adsorption of an organic film at the platinum - seawater interface', J. Mar.Res., 35, 283-291.

Neihof, R.A. and Loeb, G.I. (1972) 'The surface charge of particulate matter in seawater', Limnol. and Oceanog., 17, 7-16.

Neihof, R.A. and Loeb, G.I. (1974) 'Dissolved organic matter in seawater and the electric charge of immersed surfaces', J. Mar. Res., 32, 5-12.

Neihof, R.A. and Loeb,G.I. (1976) 'The interaction of organic dissolved matter in seawater with metallic surfaces', in V.Romanovsky (ed.), Proceedings of the 4th International Congress of Marine Corrosion and Fouling, Antibes, pp. 393-396.

O'Neill, T.B. and Wilcox, G.L. (1971) 'The formation of a "Primary Film" on materials submerged in the sea at Port Hueneme, California', Pacific Science, 25, 1-12.

Robb, I.D. (1984) 'Stereo-chemistry and function of polymers' in K.C. Marshall (ed.), Microbial Adhesion and Aggregation, Springer-Verlag, Berlin, pp. 39-49.

Rutter, P.R. and Vincent, B. (1980) 'The adhesion of micro- organisms to surfaces: physico-chemical aspects', in R.C.W.Berkeley, J.M.Lynch, J.Melling, P.R.Rutter and B.Vincent,'Microbial Adhesion to Surfaces, Ellis Horwood, Chichester, pp.79-92.

Schrader, M.E. (1982) 'On adhesion of biological substances to low energy solid surfaces', J. Coll. and Interface Sci., 88, 296-297.

Zobell, C.E. and Anderson, D.Q. (1936) 'Observations on the multiplication of bacteria in different volumes of stored seawater and the influence of oxygen tension and solid surfaces', Biological Bulletin, 71, 324-342.

MODIFICATION OF SURFACES FOR PROMOTING CELL IMMOBILIZATION

N. MOZES and P.G. ROUXHET
Unité de Chimie des Interfaces
Université Catholique de Louvain
Place Croix du Sud, 2 /18
1348 Louvain-la-Neuve
Belgium

1. Introduction

Biofilms serve beneficial purposes in the natural environment and in some modulated or engineered biological systems. For example, biofilms are responsible for the removal of dissolved and particulate contaminants in natural streams and in waste water treatment plants. Biofilms in natural water, called mats, frequently determine water quality by influencing dissolved oxygen content and by serving as a sink for toxic and/or hazardous materials. These mats may play a significant role in the cycling of chemical elements. Biofilm reactors are also used in some common fermentation processes, *eg*, the "quick" vinegar process (Characklis and Marshall, 1990).

The initial step in biofilm formation is the attachment of the "first settlers" on the support. The aim of this contribution is to show how modification of support surfaces can be used to favour such attachment.

The attachment of microorganisms to solid surfaces may, in a first analysis, be thought of as an interaction between two smooth surfaces. It must be kept in mind that surfaces usually carry an electric charge which is neutralized at a certain distance in the solution, through a diffuse double layer. Within the latter, the concentration of ions with the same sign as that of the surface charge is lower compared with their concentration in the bulk solution, and the concentration of ions with opposite sign is higher.

As two surfaces approach each other, their tendency to associate may be evaluated by the DLVO theory, which allows the computation of their interaction potential energy. Dispersion forces (London forces) contribute generally to mutual attraction. The overlap of the diffuse double layers is responsible for **electrostatic interactions** at a separation distance of several tens of nm. For surfaces carrying charge of the same sign, the existence of a potential barrier decreases the probability that their encounter will give rise to formation of a firm bond. Since microbial cells are generally negatively charged in their natural medium, a situation of attractive electrostatic interaction exists only with positively charged carriers. For example, various species were described to adsorb to ion-exchange resins (Hattori and Hattori, 1985; Wood, 1980; Durand and Navarro, 1978). Bar *et al.* (1986), who used several kinds of ion exchangers to adsorb *Acetobacter aceti*, tried to show a relation between the amount of biomass adsorbed and the charge density of the resin.

Surface energy consideration enables to compute the free energy of adhesion between two surfaces, by regarding the transformation as a replacement of two solid-liquid interfaces by one solid-solid interface. The free energy of adhesion is negative, and thus adhesion is favoured,

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 69–85.

when the surface energy of both solids is lower than that of the liquid medium; this is the case when the medium is an aqueous solution and both surfaces are hydrophobic. As interfacial free energies are computed from contact angles of liquids, their balance does not incorporate the contribution of electrostatic interactions between the two solid surfaces.

The fact that adhesion of microorganisms to various inert supports is governed by both electrostatic and hydrophobic interactions has been shown for biotechnologically relevant microorganisms (Mozes *et al.*, 1987) as well as for soil bacteria (Van Loosdrecht *et al.*, 1987a, 1987b). Electrostatic interactions act at a long distance (range of tens of nm, depending on the ionic strength) and control the rate at which surfaces can be brought close enough to form a firm bond. On the other hand, the balance of surface energies considers that, at the final state of the transformation, the two solids are in molecular contact; in a broader way, hydrophobic interactions depend on molecular organization and forces at short distances (less than 1 nm). At this stage, it must be realized that the surface of microorganisms is not smooth at a molecular level. While the overall interaction between a cell and a surface may be repulsive, **cell appendages** may bridge the distance between the cell and the surface due either to their more hydrophobic character, or their smaller radius, reducing the electric repulsion.

The picture becomes still more complicated if it is realized that the cell surface is neither smooth nor constituted of a compact solid but is made of **macromolecules** which keep a certain degree of mobility. The crucial influence of a capsule was observed in a comparison of different strains of *Acetobacter aceti* (Hermesse *et al.*, 1988). Cells having a capsule were able to adhere to various siliceous or organic supports; cells without a capsule adhered only on supports treated to decrease the negative character of their surface.

Adsorption processes may change the electrical properties and the hydrophobicity of a substratum. The adsorption of macromolecules (proteins, polysaccharides) forms a **conditioning film** on the surface. The configuration and orientation of molecules within the conditioning film are influenced by the nature of the underlying substratum; there is evidence that the properties of the latter are marked through the thickness of the conditioning film (Busscher *et al.*, 1989; Schakenraad and Busscher, 1989; J-L. Dewez, unpublished). The film of adsorbed polymers may convert the neat support/medium (solid/liquid) interface into a region of gel-like nature with which polymers or structures (*eg*, fibrils) of the cell surface may interact. Zobell and Allen, already in 1935, described the concentration and adsorption of organic matter from sea water onto various solid surfaces, and the consequent enhanced attachment and growth of bacteria. The issue has been intensively investigated since then. A recent example is the study of Beech *et al.* (1989) who identified the involvement of specific macromolecules (extracellular polysaccharides, probably as lipopolysaccharides) in the initial stage of biofilm formation on metal surfaces.

Surface microroughness may help attachment of cells (Figure 1). This may be due to the increase of the surface area available for cell-substratum contact. Moreover, cells located inside pores are sheltered from shear forces; thereby their removal rate is reduced and retention of a larger amount of cells is assured. Verrier *et al.* (1987) showed that pores and crevices at the support surface improved initial adhesion of methanogenic bacteria to various polymers. Asther *et al.* (1990) noted that adhesion of *Phanerochaete chrysosporium* to various solid carriers improved when the roughness of the latter increased. The use of porous material as carrier for cell immobilization is rather common. For example, Opara and Mann (1988) used porous bricks and Navarro and Durand (1977) used porous glass to immobilize yeast; porous glass was also used by Bücks *et al.* (1988) and Kreckeler *et al.* (1991) to immobilize bacteria. Messing and Oppermann

(1979) investigated the relevance of pore dimension for accumulating biomass; they showed that the optimum pore diameter of the support should be in the range of 4-5 times the length of the microorganism.

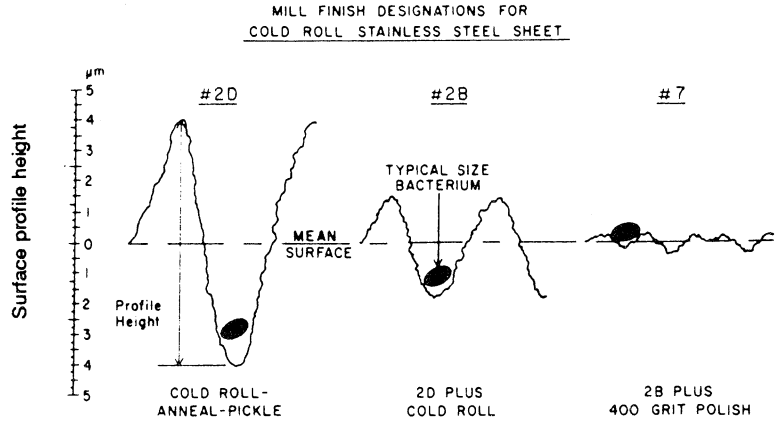


Figure 1: Diagrammatic comparison of the size of roughness on stainless steel tubing with the size of a microbial cell (adapted from Characklis, 1990).

Adequate supports for microbial cell adhesion are not always available. The main reason is that solid materials carry generally a negative surface charge, thus creating an electrostatic repulsion with microbial cells, the surface of which is also of a negative nature at neutral pH. The evident consequence is that the **substratum surface properties** must be modified in order to promote cell attachment. Acting on the electrostatic interactions is a more common practice than influencing hydrophobic interactions. Various treatments have been applied to decrease cell-support electrostatic repulsion (Rouxhet and Mozes, 1990a). These include coating the support with a layer of positively charged colloidal particles, adsorbing hydrolysable cations or polycations, grafting amine groups. For obvious reasons, treatment of the carrier surface is preferable to treatment of the cell surface.

2. Characterization of Solid Surfaces

The **chemical nature** of a surface can be analyzed by X-ray photoelectron spectroscopy (XPS). The physico-chemical properties, surface **hydrophobicity** and **electrical properties**, are studied via analyses of the wettability and the electrokinetic behaviour, respectively.

X-ray photoelectron spectroscopy is an established tool in material sciences. The technique is based on irradiating a sample by a monochromatic X-ray beam, analyzing the kinetic energy of the ejected photoelectrons and determining their binding energy in the source atom. Each peak of the recorded spectrum is characteristic of a given element. The position and the shape of the peaks are influenced by the chemical bonds and the oxidation state of the analyzed atom. The method provides thus an elemental analysis with certain information on the chemical functions. Due to inelastic scattering, the electrons collected under a peak originate from the outermost molecular layers and the analytical information concerns thus a thin layer (3-5 nm) at the surface (Rouxhet and Genet, 1991).

The **surface energy** of a solid can be estimated from measurements of contact angles of liquids and thermodynamic considerations. Various theoretical approaches for the computation have been developed, but none has yet been universally accepted. Quite often the measurement of contact angle of water alone is practiced, and the result is taken as an evaluation of surface **hydrophobicity**. High surface hydrophobicity is associated with low surface energy and vice-versa.

Various **electrokinetic phenomena** result from the fact that a surface carries an electric charge. In these phenomena, which involve the motion of a charged body relative to the surrounding fluid, the measured potential is not the electric potential at the surface of the solid body (ψ_0), but the potential at the surface of shear (ζ potential). The liquid lying between this plane and the solid surface is considered to move with the solid.

There are four different electrokinetic phenomena: electrophoresis, when a charged body is moving through a fluid due to an external electric field; electroosmosis, when a fluid is moving with respect to a charged body due to an external electric field; streaming potential, which is the potential generated when a fluid is moving with respect to a charged body due to an external force such as a pressure difference; sedimentation potential, which is generated when a charged body is moving through a fluid due to an external force, such as gravity.

Micro-electrophoresis is by far the most common procedure for determining ζ potential of solids; its use is limited to suspensions of finely divided colloidal particles. Doren *et al.* (1989) have used the microelectrophoresis apparatus of Pen Kem Inc. (Laser Zee model 500) to determine the ζ potential of plates. They modified the standard rectangular electrophoretic chamber and fitted plates of the solid to be analyzed in such a way that they formed the upper and lower walls of the chamber. The analysis of the mobility profile of a probe colloid through the thickness of the chamber allowed determination of the electroosmotic mobility of the liquid (10 mM KNO_3 solution), which is directly related to the ζ potential of the walls. Figure 2 illustrates the use of this

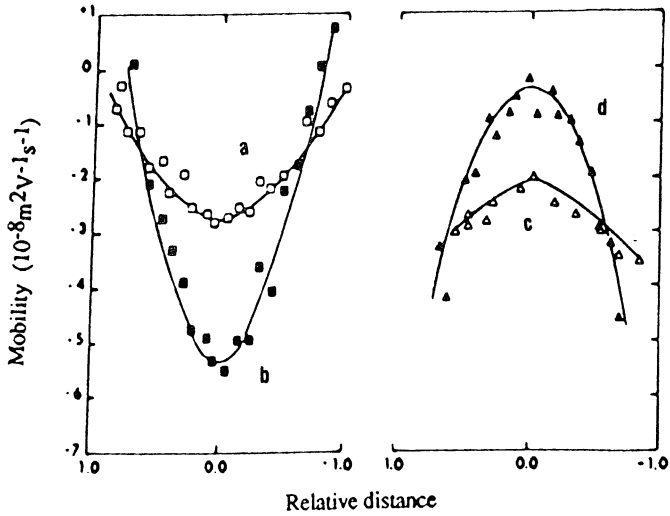


Figure 2: Variation of the mobility of latex particles as a function of the relative distance with respect to the center of the chamber; walls made up by polycarbonate plates oxidized (b, d) or not (a, c), submitted (c, d) or not (a, b) to treatment by ferric nitrate solution (adapted from Changui *et al.*, 1987).

technique for investigating the influence of oxidation and deposition of ferric hydroxide on polycarbonate. Commercial instruments for streaming potential measurements are less common than those for microelectrophoresis. One measures the potential difference built across a capillary, a slit or a plug through which a liquid is forced in laminar flow under a given pressure gradient (Wagenen and Andrade, 1980). Thereby the surface electrical properties of plates, fibers or particles can be investigated.

3. Coating a Support by Positively Charged Colloidal Particles

The adhesion of positively charged colloidal particles (hematite, Fe_2O_3; hydrous alumina, $Al(OH)_3$) is achieved by allowing an aqueous suspension of the particles to settle onto the support, and then removing the non-adhering particles by washing with a laminar flow of water.

A typical protocol starts with the preparation of the sol (Matijevic and Scheiner, 1978; Kayem and Rouxhet, 1983). The concentrated stock solution is diluted to a concentration in the range of 10^9 particles/ml. The pH is adjusted to about 7-8 for alumina or 4-6 for hematite by careful addition of NaOH. If flocculation occurs, sonication helps to disperse the suspension. The colloid suspension is poured over the support and left in contact for about 20 h. The support is then rinsed with a stream of demineralized water. If the treated support is not to be used immediately, it can be stored wet. Cell suspension (1 to 5 • 10^8 cell/ml) is brought in contact with the treated carrier; after about 2 h, non-adhering cells are washed away by a stream of water.

The electrostatic attraction between particles and charged support leads to rapid adhesion of the first sedimenting particles. However, this tends to reverse the overall charge of the substratum and late arriving particles are partly repelled by the already adhering ones. Formation of a dense layer may require long contact times and sedimentation of a large excess of particles (Rouxhet *et al.*, 1987).

Figure 3 shows the density of adhering hematite and alumina particles as a function of the amount sedimented (present on the support before washing). The maximum density of one layer of hematite particles (rounded edged cubic particles with edge length of 0.5μm) in square close packed array would be about 4 • 10^9 particles/cm^2. For the alumina particles (spheres of 0.25μm diameter) in hexagonal close packed array it would be 2 • 10^9 particles/cm^2. The maximum coverage obtained for both colloids was close to the maximum allowed for a monolayer, but required sedimentation of two to three times more particles.

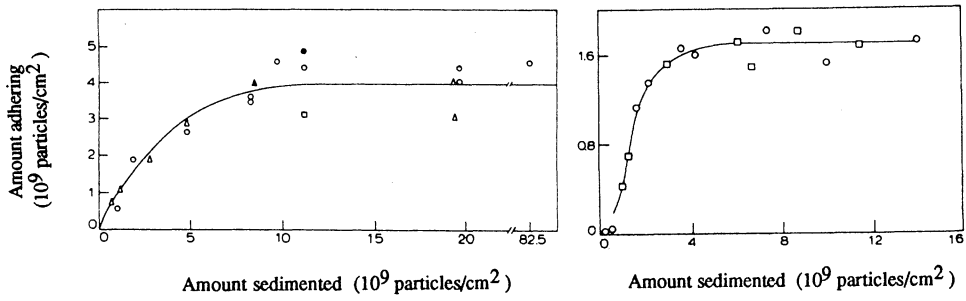


Figure 3: Variation of the density of colloid particles adhering on glass, as a function of the amount of particles sedimented: left, hematite ; right, alumina.

The use of colloidal oxides to promote adhesion was first studied in the context of adhesion of yeast cells. It was shown that the immobilized cells retained their capacity to convert glucose to ethanol (Mozes and Rouxhet, 1985). The same procedure was also used for achieving immobilization of bacteria, *Xanthomonas campestris* (unpublished) and *Arthrobacter simplex* (Mozes and Rouxhet, 1984). The latter was immobilized on glass beads treated by $Al(OH)_3$ in a reactor which operated during 30 days, performing 10 repeated runs of complete transformation of cortisol to prednisolone.

The procedure of coating a support by positively charged colloidal particles is generally simple to perform and the results are rather reproducible. However, the need to prepare the sol, which is not commercially available, may be considered as a drawback. A low-cost commercial source of adequate colloids would give this method a promising future.

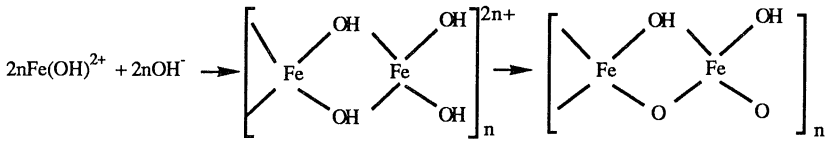
4. Adsorption of Hydrolysable Ions

Multivalent cations such as Fe^{3+} and Al^{3+} react in water and form polynuclear species by association of several metal ions with oxygen and hydroxide ligands. The concentration of the salts, the nature of the anion, the pH, the temperature and the age of the solution govern the formation of the polynuclear ions.

4.1. FERRIC IONS

Dissolution of ferric salts in water generates rapidly mononuclear hydroxy-complexes (1) and, more slowly, polynuclear species (2).

$$Fe^{3+} + H_2O \rightarrow Fe(OH)^{2+} + H^+ \qquad (1)$$



(2)

At low pH (typically, pH below 2.5 for 1 mM Fe^{3+} solution), adsorption of mononuclear ions on a silica surface occurs by reaction with SiOH groups and formation of $SiO\text{-}Fe(OH)^+$. The quantity fixed is strongly dependent on the pH: it is low at the isoelectric point (iep) of silica (pH about 2) and increases as the pH rises. Only a small proportion of the superficial SiOH groups reacts, the adsorption being limited by the rising surface potential. If no acid is added to the ferric solution, polynuclear ions are formed which represent a metastable state with respect to the formation of oxy-hydroxide. The presence of a solid surface accelerates the process and induces the precipitation of oxy-hydroxide, which then forms a coating on the surface. When a freshly prepared salt solution is allowed to age in contact with the solid surface a coating is achieved more readily than when the pH of a solution is raised abruptly, leading to a quick precipitation.

A typical protocol consists of preparing a 1.8 mM solution of $Fe(NO_3)_3$ in water, the pH of which is 2.8-2.9. The carrier is immersed in a freshly prepared solution at room temperature and left overnight. During that time, the originally clear solution turns yellow, indicating the formation of polynuclear species. The carrier is taken out of the solution, rinsed well and used immediately

or kept wet (immersed in water) for later use. Bringing a cellular suspension in contact with a carrier treated in this way results in adhesion of the cells to the support surface.

The oxy-hydroxide formed at the surface of the carrier makes it less negative. Figure 4 presents the electrophoretic mobility of fine glass particles before and after treatment with ferric nitrate solution. The isoelectric point (iep) of the glass is shifted from 2 to 4.5-5 due to the treatment (Hermesse *et al.*, 1988).

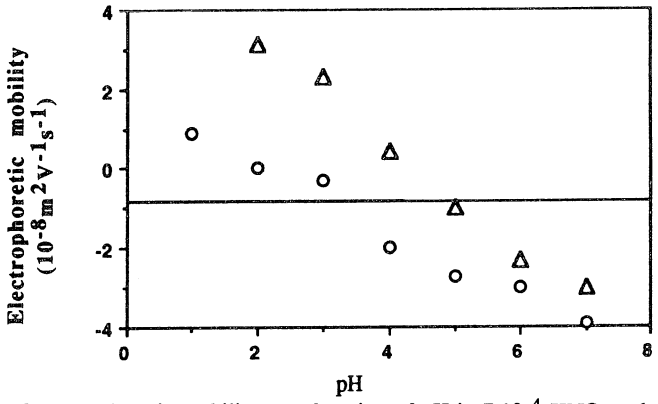


Figure 4: Variation of electrophoretic mobility as a function of pH in 7.10^{-4} KNO_3 solution: O, glass particles; Δ glass particles treated by a solution of ferric nitrate (adapted from Hermesse *et al*, 1988).

Changui *et al.* (1987) investigated the influence of the treatment of native and oxidized bis-phenol A polycarbonate (PC) by solutions of ferric nitrate as described above. Table 1 presents the results of characterization of the treated PC surface by conventional chemical analysis and XPS analysis, as well as the water contact angle and the ζ potential measured by the modified microeletrophoresis apparatus described by Doren *et al.* (1989), (Figure 2). The distribution of iron on the surface was evaluated by comparing results of XPS analysis and chemical analysis, through various models. It was concluded that iron oxy-hydroxide, in the form of about 10 nm particles, was coating the surface. The deposition of ferric hydroxide particles provoked a charge reversal which allowed adhesion of a dense layer of yeast cells. When the ferric treatment was applied to native PC, subsequent drying prevented adhesion of a dense layer of cells. This did not occur when the PC surface had been oxidized before the ferric treatment, due to lower surface hydrophobicity of the support and better anchorage of the ferric hydroxide particles on its strongly negative surface.

Such treatment with a solution of ferric nitrate was successfully applied to glass and various polymeric materials that served then as carriers for immobilization of *Saccharomyces cerevisiae* (Mozes *et al.*, 1987), *Acetobacter aceti* (Mozes *et al.*, 1987; Hermesse *et al.*, 1988), *Bacillus licheniformis* (unpublished), and a series of anaerobic bacteria (Fregard, 1991). A few examples are shown in Figure 5a,b. In these tests the cells were suspended in water. When cells of *A. aceti* were suspended in their culture medium, they failed to adhere to treated glass. It was shown that that was due to reconditioning of the ferric-treated surface by adsorption of constituents of yeast extract.

Table 1. Surface properties of bisphenyl-A polycarbonate submitted or not to oxidation and Fe treatment; influence on the adhesion of *Saccharomyces cerevisiae* (Changui *et al.* , 1987).

Support	N-N (a)	N-Fe (a)	Ox-N (a)	Ox-Fe (a)
Amount of iron (μmol/m^2) (b)	0	26	0	55
XPS Analysis (c)				
O/C	0.19	0.29	0.35	0.65
Fe/C	0.00	0.03	0.00	0.05
S/C	0.00	0.00	0.05	0.10
Water contact angle (°) (d)	77	86	55	40
Zeta potential (mV) (e)	-26	+18	-87	+75
Adhesion of yeast (f)	none	dense	none	dense and uniform

(a) N-N : nontreated; N-Fe : immersed 24 h in 1.8 mM ferric nitrate solution at pH 4; Ox-N : immersed 30 min in sulfo-chromic mixture; Ox-Fe : Ox-N subsequently immersed 24 h in 1.8 mM ferric nitrate solution at pH 4.

(b) Amount of ferric hydroxide retained by the surface, determined by chemical analysis.

(c) Apparent surface composition determined by X-ray photoelectron spectroscopy and expressed in terms of atom concentration ratios.

(d) Water contact angle measured by the sessile drop method.

(e) Apparent zeta potential determined by electroosmotic technique.

(f) Adhesion tests performed with *Saccharomyces cerevisiae* suspended in HNO_3 solution of pH 3 or 5.

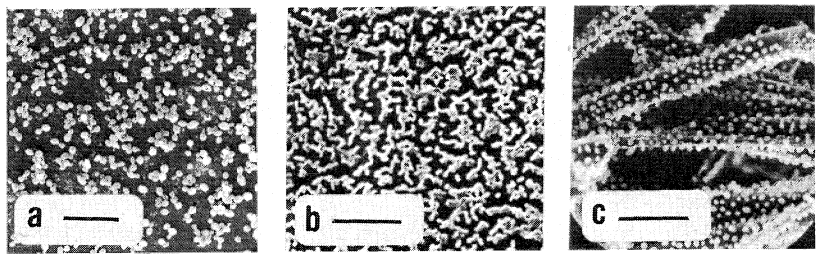


Figure 5: Cells immobilized on treated carriers: **a**, *Saccharomyces cerevisiae* on polyamide treated by ferric nitrate solution, bar represents 20 μm; **b**, *Acetobacter aceti* on polycarbonate treated by ferric nitrate solution, bar represents 5 μm; **c**, *Kluyveromyces lactis* on glass fiber treated by chitosan solution, bar represents 20 μm.

4.2. ALUMINIUM IONS

The Al^{3+} ion is coordinated with six molecules of water. The high charge of the Al^{3+} ion weakens the OH bond, as a result a proton is released and the formation of polynuclear cations may take place.

A typical protocol for treatment of a support with aluminium ions is the following. Glass plates are immersed in an aqueous solution of $Al(NO_3)_3$ 0.1 mM and KNO_3 10 mM. The pH, initially 4,

is raised progressively up to 6 by slow addition of KOH during 30 min. The plates are kept in the solution 15 minutes longer, then rinsed with a solution of KNO_3 10 mM pH 6. The electrokinetic properties of glass plates treated in this way were compared with those of non-treated plates: the electroosmotic mobility at pH 6 was $-3.4 \cdot 10^{-8}$ $m^2V^{-1}s^{-1}$ for non-treated glass, and $+4.5 \cdot 10^{-8}$ $m^2V^{-1}s^{-1}$ for Al-treated glass (Doren *et al.*, 1989).

Glass plates treated with a similar procedure were used successfully for immobilization of *Xantomonas campestris* and *Escherichia coli* (Rouxhet and Mozes, 1990a).

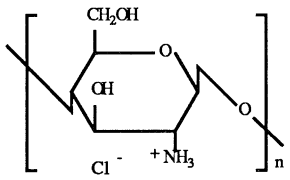
The use of hydrolysable ions for rendering the surface less negative is a very simple procedure and requires inexpensive reagents. A serious limitation for the treatment with hydrolysable ions is the large amount of acid wastes which render it impractical for large-scale operation.

5. Adsorption of Organic Polycations

Polymers may adsorb firmly onto surfaces as a result of a large number of weak bonds. Adsorption of cationic polymers is enhanced due to electrostatic interactions with the negative surface. The strongly adsorbed polycationic molecule still leaves free cationic sites which may interact with anionic sites of the cell surface. The cationic site is a quaternary ammonium or a protonated amine; in the later case its positive charge is pH dependent.

5.1. CHITOSAN {POLYGLUCOSEAMINE, (1,4)-2AMINO-2DEOXY-β-D-GLUCAN}

This is a deacetylated derivative of chitine. As it is obtained by hydrolysis of a natural product and since the deacetylation is not always complete, the chain length and the number of positive charges per molecule are not regular.



A stock solution is prepared by dissolution of 5 g flakes of chitosan in 1 l of 1M acetic acid, and either used as such (pH 3.1) or dialyzed against deionized water until a pH of about 6.3 is obtained. This stock solution is diluted with water to a final concentration of 0.4 to 1g/l . The carrier is immersed during 1 h at room temperature in this solution (Champluvier *et al.*,1989a).

The effect of chitosan treatment (immersion during 1 h in non-dialyzed solution, then drying) on the surface charge of tissue-culture-grade polystyrene was demonstrated by a change of electroosmotic mobility at pH 5.2 from $-8.2 \cdot 10^{-8}$ to $+5.4 \cdot 10^{-8}$ $m^2V^{-1}s^{-1}$ (Doren *et al.*, 1989). Chitosan treatment of glass plates, glass fibers and polyurethane foam was successful in promoting adhesion of yeast (*Kluyveromyces lactis*) and bacteria (*Klebsiella oxytoca, Bacillus licheniformis*). An example is illustrated by Figure 5c.

In order to investigate the influence of experimental conditions, adhesion tests were performed by sedimenting cells of *Kluyveromyces lactis* from suspension in water onto glass plates pretreated by chitosan solution and rinsing the plates with water in order to remove non-adhering cells

(Champluvier *et al.*, 1989a). Three alternative procedures were compared after immersion of the plate in the chitosan solution: the solution was evaporated at 100°C; the support was drained to remove the excess solution and not dried; the support was rinsed with distilled water and not dried. If the glass plates were pretreated by a nondialyzed (low pH) chitosan solution, a dense coverage by a single layer of cells was achieved with no appreciable differences among the three procedures. When glass was pretreated with a dialyzed chitosan solution (pH 6.3), adhesion of multilayers or flocks was detected for the two procedures which did not involve drying. The formation of multilayers is attributed to desorption of the polycation from glass and its adsorption on the cells, leading to their association. The absence of multilayers when the support was treated by evaporating the chitosan solution is attributed to a dehydration of chitosan which slows its dissolution. The fact that the use of a dialyzed solution of chitosan favours the formation of multilayers, and a non-dialyzed solution does not, is probably due to the lower chitosan solubility at the higher pH, which leads to a larger adsorbed amount.

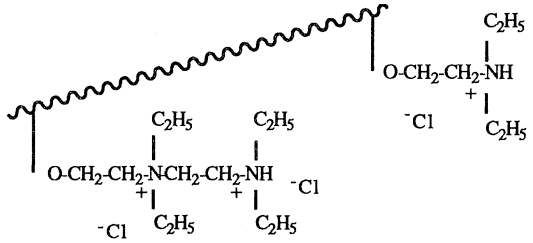
Additional tests were performed with glass plates pretreated by evaporation of a nondialyzed chitosan solution; the medium in which the cells were suspended and the contact time were varied. When the cells were suspended in water, multilayers were not observed after 1 h (the standard duration) of contact between the cell sediment and the support but were found after 12 h of contact. Sedimentation of cells suspended in yeast extract solution at pH 6.5 provided less dense, less uniform and less reproducible cell coverage, compared with cells suspended in water. This was attributed to the fact that adsorption of constituents of the culture medium modified the surface properties created by the chitosan treatment. The same was observed with cells suspended in yeast extract solution at pH 4; however in this case cell aggregates were also observed when the contact between the cell sediment and the support was extended to 12 h. These observations indicate again a cell aggregation caused by a redissolution of previously deposited chitosan, which is more pronounced at pH 4 than pH 6.5.

These results show that the procedures used to pretreat the support and to bring the cells in contact with its surface determine the distribution of the cells retained on the surface: dense single layer, multilayer, isolated cell aggregates.

Permeabilized yeast cells of *Kluyveromyces lactis* in which lactase was confined were coimmobilized with *Klebsiella oxytoca* cells in a chitosan treated fiberglass matrix (Champluvier *et al.*, 1989b). The permealibilized yeast converted lactose into glucose and galactose, which were assimilated by the bacteria to produce 2,3-butanediol. The stability of the coimmobilized system was demonstrated in a microreactor by the continuous conversion of lactose into 2,3-butanediol at 30°C during 25 days. Compared to the literature data on direct conversion of lactose using pure cultures, higher butanediol concentrations were obtained and 10 to 100 times higher rates of production were achieved. *B. licheniformis* immobilized in the same way could also maintain metabolic activity; in that case the product was the antibiotic bacitracin (unpublished).

5.2. DEAE-DEXTRAN

Dextrans are homopolymers of D-glucopyranose units connected by glycosidic linkage. The main chain consists of α(1-6) linkages with side chains attached by α(1-3) linkages. Such polymers are produced by several species of bacteria. They are naturally extremely polydisperse; controlled acid hydrolysis followed by careful fractionation is used to produce dextran fractions with narrow molecular weight range. The radical diethyl aminoethyl (DEAE) may be grafted on such polymers as shown below.



By immersion of a glass carrier in a 1% solution of DEAE-dextran in water during 20 min, one could obtain a reversal of the surface charge and an increase of surface hydrophobicity. The electroosmotic mobility at pH 7 changed from $-5.8 \cdot 10^{-8}$ $m^2V^{-1}s^{-1}$ for naked glass, to $+4.1 \cdot 10^{-8}$ $m^2V^{-1}s^{-1}$ for DEAE-dextran treated glass, and the water contact angle changed from 15° to 81° respectively (unpublished).

This treatment makes the glass carrier very attractive for microbial cells. Bücks *et al.* (1988) immobilized *Corynebacterium glutamicum* cells on DEAE-dextran treated porous glass and used them for conversion of α-ketocaproic acid to l-leucine. In the authors' laboratory *Bacillus licheniformis* was immobilized in this manner on glass fibers and polyurethane foam.

5.3. POLYETHYLENIMINE (PEI)

This polymer is commercially available, inexpensive and non-toxic.

$$-[CH_2\text{-}CH_2\text{-}NH\text{-}]_n$$

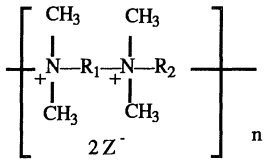
Its use was described by D'Souza *et al.* (1986). A 0.2% aqueous solution at pH 7 is brought in contact with the support during 2 h, the excess solution is drained and the carrier dried. The drying step is essential. Contact of a yeast suspension with a plate treated in this manner resulted in a dense uniform monolayer of adhering cells. The attachment was strong and resisted extreme pH conditions or changes in ionic strength. The viability of the cells was not affected, and the enzymatic activity of their invertase could be exploited in a small reactor.

PEI was also used with cotton cloth as a carrier to immobilize ureolytic acetobacter species (Kamath and D'Souza, 1991). Cotton cloth was treated with PEI and dried; then it was soaked in a suspension of washed cells and the non-adhering cells were removed by rinsing with water. Combination of adhesion of cells onto the treated support and their flocculation by free molecules of PEI led to retention of higher amounts of cells. The immobilized bacteria retained 80% of their original ureolytic activity even after 10 cycles of repeated batch runs. They have also been used in a continuous reactor with a half life time of 15 days.

5.4. POLYCATIONS DESCRIBED FOR PROMOTION OF ANIMAL CELL ADHESION

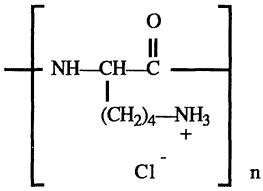
Adsorption of polycations is often described as a means to promote adhesion of animal cells to glass or polystyrene surfaces. Some of the polycations reported in the literature are listed below.

Ionenes are long linear polymers of molecular weight between 10.000 and 60.000 with quaternary ammonium at regular intervals. Their general formula is:

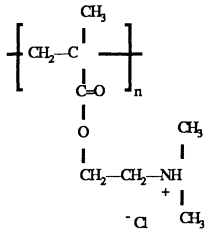


R_1, R_2 are aliphatic, aromatic or heterocyclic groups; Z is an anion, generally halide. An example of common ionene is polybrene where $R_1 = (CH_2)_6$, $R_2 = (CH_2)_3$ and $Z = Br^-$. Ionenes are used at very low concentrations (2-4 mg/l) and must be washed away after contact with the support as they may be toxic to animal cells.

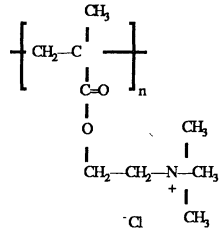
Polylysine is also used as relatively diluted solution. It adsorbs instantaneously, and a contact of 5 minutes with the carrier is sufficient. This reagent is also toxic for animal cells and its excess must be removed before the cells are added.



Copolymers of *hydroxyethyl methacrylate* (HEMA) and *dimethylaminoethyl methacrylate-hydrochloride* (DMAEMA-HCl) or *trimethylaminoethyl methacrylate-chloride* (TMAEMA-Cl) were used by Hattori *et al.* (1985). Streaming potential measurements and bulk titration carried out in parallel to adhesion tests of fibroblasts, clearly demonstrated that cell attachment and proliferation were dependent on the mole fraction of cationic molecules of the copolymer and on the ζ potential of the treated support.



DMAEMA-HCl



TMAEMA-Cl

The main advantage of using polycations is that their interactions with surfaces are stable with time. They confer a positive nature to the carrier, sometimes even independently of the pH. This is of great importance for systems where the metabolic activity results in alkalinization of the medium. The polymers adsorb strongly to the support and make it attractive for cells. Excess of polymers may provoke cell flocculation that result in retention of very high amount of cells. Care

must be taken to eliminate nonadsorbed polycation before animal cells are brought in contact with treated carriers, since they disrupt the plasma membrane.

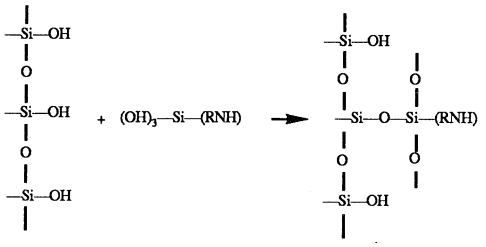
6. Grafting Amine Functions

Covalent binding of positively charged molecular entities, like amine at adequate pH, can also confer a positive nature to the support surface.

Triaminosilane {N'-(β-aminoethyl)-N-(β-aminoethyl)-γ-aminopropyl-trimethoxysilane}, $(OCH)_3$ Si $CH_2(CH_2CH_2NH)_2$ $CH_2CH_2NH_2$, is protonated at neutral pH. On the other hand, the methoxy groups hydrolyse in water (3).

$$(OCH_3)_3\text{-Si-(RNH)} + 3H_2O \rightarrow (OH)_3\text{-Si-(RNH)} + 3\,CH_3OH \qquad (3)$$

The product condenses (4) and may react with any available hydroxide group of a support (eventually glass).



(4)

Condensation of hydroxide groups of adjacent silane molecules and cross-linking take place upon curing. The result is a carrier with firmly bound hydrophobic material carrying a positive charge at neutral pH.

A typical protocol is the following. A triaminosilane (A 1130 Union Carbide) solution of 1% in water (pH 10.5) is acidified to about pH 5 with acetic or hydrochloric acid. Glass plates are immersed in this solution at room temperature during 2 h. They are then drained, but not rinsed, and cured in an oven (120°C) during 2 h. The cooled plates can be used immediately or stored dry.

The inversion of the surface potential was demonstrated by electroosmotic mobility measurements. Values obtained for native glass plates and aminosilane treated glass plates were $-3.50 \cdot 10^{-8}$ and $+ 2.36 \cdot 10^{-8}$ $m^2V^{-1}s^{-1}$, respectively. The increased hydrophobicity was noted by determination of water contact angles; the measured values were 20° and 60° respectively.

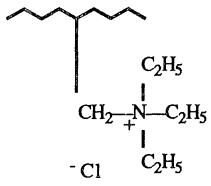
An alternative protocol was described by Bücks *et al.* (1988) who used open-pore sintered glass as a carrier. The triaminosilane (A 1130) solution (5%) was made in methanol and the pH adjusted to 5 with HCl. The carrier was boiled for 4 h under reflux in that solution, then rinsed with water for 4 h and heated at 120°C overnight. Rinsing before curing reduced the extent of polymerization and cross-linking and hence the risk of obstructing the carrier pores.

When a suspension of microorganisms is brought in contact with the treated carrier, the cells adhere at the surface and do not detach even after rinsing with an intensive stream of water.

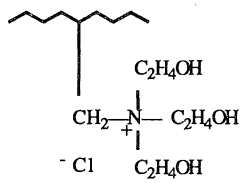
A matrix of glass wool coated with phenolic resin was treated by triaminosilane in a manner similar to the standard protocol described above. The streaming potential at pH 6 to 7 which was

originally -11 mV became +5 mV after the treatment. Hydrophilic cells (like yeast *S. cerevisiae* or the bacteria *Acetobacter aceti*) were not retained by the matrix unless it was treated with the aminosilane. On the other hand, hydrophobic cells, like *Moniliella pollinis* and *Kluyveromyces fragilis*, did adhere to the non-treated glass wool (unpublished). *S. cerevisiae* was also immobilized by Navarro and Durand (1977) on silica treated by aminosilane. *Bacillus licheniformis* was immobilized in view of production of the antibiotic bacitracin: a dense homogeneous coverage was obtained after 2 h of contact of a triaminosilane-treated glass plate with the cell suspension; a very good adhesion was also obtained on the fibers of triaminosilane-treated glass wool after circulation of the bacterial suspension through the matrix (unpublished). *Corynebacterium glutamicum* , immobilized on porous glass material treated by triaminosilane, was used for conversion of α-ketoisocaproate to l-leucine (Bücks *et al.* 1988).

Another method to confer a positive charge to a support surface and promote cell adhesion is grafting a quaternary ammonium compound. The surface charge in this case is positive, independent of the pH. Thus triethylamine and triethanolamine were grafted on polystyrene (PS) in solution. The resin was then precipitated and used as a carrier for immobilization of *S. cerevisiae*.



Triethylamine - PS



Triethanolamine - PS

Very dense adhesion was obtained in both cases. The production of ethanol by the yeast immobilized on triethanolamine-grafted PS was similar to that by free cells in suspension. On the other hand, the yeast immobilized on triethylamine-grafted PS suffered severe reduction of activity. The reason was found to be the release of free triethylamine that remained unbound in the resin and is toxic for the yeast (Rouxhet and Mozes, 1990b).

Covalent binding is much stronger than adsorption; therefore grafting is regarded as an irreversible alteration of the surface properties.

7. Physical Treatments

Adhesion of animal cells to solid surfaces is beyond the scope of this chapter. It is however interesting to mention the basic approach in the efforts to enhance adhesion (to be followed by proliferation and differentiation) of these cells. It has been shown that the surface free energy (γ_s) or hydrophobicity of the substratum is a key factor in this respect (Schakenraad and Busscher, 1989). Radio frequency plasma discharge is currently practiced to alter the surface properties of polymers by introducing reactive or functional groups. Basically, it creates active species, the nature of which depends on the experimental set up and conditions, and which react with the polymer surface. Introduction of functional groups like -OH, -C=O, -COOH, $-NH_2$, increases γ_s (Dewez *et al.*, 1990) and favours adhesion of epithelial (Kirkpatrick *et al.*, 1991) and endothelial

(Dewez *et al.*, 1990) cells. In optimal conditions for the cells (presence of proteins), their attachment is independent of the nature of the functional groups at the polymer surface, as long as γ_s is high enough. However, if the cellular protein synthesis is blocked, it seems that attachment of epithelial cells takes place only when the discharge had conferred a positive nature to the support surface. It has been shown recently that the surface of polyethyleneterephtalate (PET) became an excellent support after treatment by NH_3-plasma. The water contact angles of the non-treated and treated materials were 66° and 32° respectively; the iep, obtained from streaming potential measurements, were 2 and 4 respectively. Epithelial cells adhered on the treated PET much faster than on the non-treated PET, and a confluent cell monolayer was established only on the treated material (J-L. Dewez, personal communication).

8. Concluding Remarks

Various methods for modification of the surface electric properties of supports in order to promote cell adhesion were described. The list is of course incomplete and other possibilities exist. Altering surface energy (increase hydrophobicity to favour microbial adhesion, increase hydrophilicity to favour anchorage and proliferation of animal cells) can also be considered.

The methods described here have been established and demonstrated in laboratory-scale experiments. Their application in large scale operations must take into consideration the ecological impact of manipulating large volumes of reagents. Constituents of the culture medium may mask the positive charge conferred to the carrier surface. Some techniques combine adhesion and flocculation, ensuring thus a rapid accumulation of biomass at the substratum surface; polycations seem to be most suitable in this respect. Applicability of the techniques based on coating with colloidal particles depends on availability of cheap sols.

Among factors that should be considered for selecting a method, one may cite: reversibility of the surface modification, influence of sterilization on the modified surface, eventual toxicity of the reagents used, price of the necessary reagents and conservation of the suited activity.

Acknowledgement

The authors' laboratory is affiliated with the Faculty of Agricultural Sciences; P.G.R. is a member of the Research Center for Advanced Materials. Many of the works described in this review were realized thanks to financial support of the Departement of Education and Scientific Research (Concerted Action of Physical Chemistry of Interfaces in Biotechnology) and of the European Community (Biomolecular Engineering Programme and Biotechnology Action Programme).

References

Asther, M., Bellon-Fontaine, M.N., Capdeville, C. and Corrieu, G. (1990), 'A thermodynamic model to predict *Phanerochaete chrysosporium* INA-12 adhesion to various solid carriers in relation to lignin peroxidase production', Biotechnol. Bioeng., **35**, 477-482.

Bar, R., Gainer, J.L. and Kirwan, D.J. (1986), 'Immobilization of *Acetobacter aceti* on cellulose ion exchangers: Adsorption isotherms', Biotechnol. Bioeng., **28**, 1166-1171.

Beech, I.B. and Gaylarde, C.C. (1989), 'Adhesion of *Desulfovibrio desulfuricans* and *Pseudomonas fluorescens* to mild steel surfaces', J. Appl. Bacteriol., **67**, 201-207.

Büchs, J., Mozes, N., Wandrey, C. and Rouxhet, P.G. (1988), 'Cell adsorption control by culture conditions. Influence of phosphate on surface properties, flocculation and adsorption behaviour of *Corynebacterium glutamicum*', Appl. Microbiol. Biotechnol., **29**, 119-128.

Busscher, H.J., Weerkamp, A.H., Van Der Mei, H.C., Van Steenberghe, D., Quirynen, M., Pratt, I.H., Marechal, M. and Rouxhet, P.G. (1989), 'Physico-chemical properties of oral streptococcal cell surfaces and their relation with adhesion to solid substrata in vitro and in vivo', Colloids and Surfaces, **42**, 345-353.

Champluvier, B., Marchal, F. and Rouxhet, P.G. (1989a), 'Immobilization of lactase in yeast cells retained in a glass wool matrix', Enzyme Microb. Technol., **11**, 422-430.

Champluvier, B., Francart, B. and Rouxhet, P.G. (1989b), 'Co-immobilization by adhesion of ß-galactosidase in nonviable cells of *Kluyveromyces lactis* with *Klebsiella oxytoca*: conversion of lactose into 2,3-butanediol', Biotechnol. Bioeng., **34**, 844-853.

Changui, C., Doren, A., Stone W.E.E., Mozes, N. and Rouxhet, P. (1987), 'Surface properties of polycarbonate and promotion of yeast cells adhesion', J. Chim. Phys., **84**, 276-281.

Characklis, W.G., 'Biofilm processes', in: W.G. Characklis and K.C. Marshall (eds.), Biofilms, John Wiley & Sons, Inc., New York, Chichester, Brisbane, Toronto, Singapore, ch. 7, pp. 195-231.

Characklis, W.G., Marshall, K.C. (1990), 'Biofilms: A basis for an interdisciplinary approach', in: W.G. Characklis and K.C. Marshall (eds.), Biofilms, John Wiley & Sons, Inc., New York, Chichester, Brisbane, Toronto, Singapore, ch. 1, pp. 3-15.

Dewez, J.L., Doren, A., Schneider, Y.J., Legras, R. and Rouxhet, P.G. (1991), 'Surface study of new microporous polymeric membranes used as a substratum for animal cell culture', in: P. Grange and B. Delmon (eds.), Interfaces in new materials, Elsevier Applied Science, London and New York, pp. 84-94.

Doren, A., Lemaître, J. and Rouxhet, P.G. (1989), 'Determination of the zeta potential of macroscopic specimens using microelectrophoresis', J. Colloid Interface Sci., **130**, 146-156.

D'Souza, S.F., Melo, J.S., Deshpande, A. and Nadkarni, G.B. (1986), 'Immobilization of yeast cells by adhesion to glass surface using polyethylenimine', Biotechnol. Lett., **8**, 643-648.

Durand, G. and Navarro, J.M. (1978), 'Immobilized microbial cells', Process Biochemistry, September 1978, 14-23.

Fregard, F. (1991), 'Role des interactions hydrophobes et électrostatiques dans l'adhésion de bactéries méthanogènes aux matériaux de faible énergie de surface', PhD Thesis, Université des Sciences et Techniques de Lille I (France).

Hattori, R. and Hattori, T. (1985), 'Adsorptive phenomena involving bacterial cells and an anion exchange resin', J. Gen. Appl. Microbiol., **31**, 147-163.

Hattori, S., Andrade, J.D., Hibbs, J.B., Gregonis, D.E. and King, R.N. (1985), 'Fibroblast cell proliferation on charged hydroxyethyl methacrylate copolymers', J. Colloid Interface Sci., **104**, 72-78.

Hermesse, M.P., Dereppe, C., Bartholomé, Y. and Rouxhet, P.G. (1988), 'Immobilization of *Acetobacter aceti* by adhesion', Can. J. Microbiol., **34**, 638-644.

Kamath, N. and D'Souza S.F. (1991), 'Immobilization of ureolytic cells through flocculation and adhesion on cotton cloth using polyethylenimine', Enzyme Microb. Technol., **13**, 935-938.

Kayem G.J., Rouxhet P.G. (1983), 'Adsorption of colloidal hydrous alumina on yeast cells', J. Chem. Soc., Faraday Trans., **79**, 561-569.

Kirkpatrick, C.J., Mueller-Schulte, D., Royé, M., Hollweg, G., Gossen, C., Richter, H. and Mittermayer, C., 'Surface modification of polymer to permit endothelial cell growth', Cells and Materials, **1**, 93-108.

Krekeler, C., Ziehr, H. and Klein, J. (1991), 'Influence of physicochemical bacterial surface properties on adsorption to inorganic porous supports', Appl. Microbiol. Biotechnol., **35**, 484-490.

Matijevic, E. and Scheiner, P. (1978), 'Ferric hydrous oxide sols. III. Preparation of uniform particles by hydrolysis of Fe(III)-chloride, -nitrate, and -prechlorate solutions', J. Colloid Interface Sci., **63**, 509-524.

Messing, R.A. and Oppermann, R.A. (1979), 'Pore dimensions for accumulating biomass. I. Microbes that reproduce by fission or by budding', Biotechnol.Bioeng., **21**, 49-58.

Mozes, N. and Rouxhet, P.G. (1984), 'Dehydrogenation of cortisol by *Arthrobacter simplex* immobilized as supported monolayer', Enzyme Microb. Technol., **6**, 497-502.

Mozes, N. and Rouxhet, P.G. (1985), 'Metabolic activity of yeast immobilized as supported monolayer', Appl. Microbiol Biotechnol., **22**, 92-97.

Mozes, N., Marchal, F., Hermesse, M.P., Van Haecht, J.L., Reuliaux, L., Léonard, A.J. and Rouxhet, P.G. (1987), 'Immobilization of microorganisms by adhesion: Interplay of electrostatic and nonelectrostatic interactions', Biotechnol. Bioeng., **30**, 439-450.

Navarro, J.M. and Durand, G. (1977), 'Modification of yeast metabolism by immobilization onto porous glass', European J. Appl. Microbiol., **4**, 243-254.

Opara, C.C. and Mann, J. (1988), 'Development of ultraporous fired bricks as support for yeast cell immobilization', Biotechnol. Bioeng., **31**, 470-475.

Rouxhet, P.G. and Mozes, N. (1990a), 'Physical chemistry of the interface between attached micro-organisms and their support', Wat. Sci. Tech., **22**, 1-16.

Rouxhet, P.G. and Mozes, N. (1990b), 'The micro-environment of immobilized cells: critical assessment of the influence of surfaces and local concentrations', in Physiology of Immobilized Cells (J.A.M. de Bont, J. Visser, B. Mattiasson and J. Trampers, eds.), Proc. Int. Symp. Wageningen, The Netherlands, 10-13 December 1989, Elsevier Science Publishers, Amsterdam, pp. 343-354.

Rouxhet, P.G. and Genet, J.G. (1991), 'Chemical composition of the microbial cell surface by X-ray photoelectron spectroscopy', in: N. Mozes, P.S. Handley, H.J. Busscher and P.G. Rouxhet (eds.), Microbial cell surface analysis: structural and physico-chemical methods, VCH, New York, ch.8 pp.173-220.

Rouxhet, P.G., Mozes, N., Hermesse, M.P. and Matta-Ammouri, G. (1987), 'Immobilization of microorganisms by adhesion to a support: influence of physico-chemical factors', in Proc. 4th European congress on Biotechnol. (O.M. Neijssel, R.R. van der Meer and K.Ch.A.M. Luyben, eds.), Elsevier Science Publishers, Amsterdam, pp. 193-196.

Schakenraad, J.M. and Busscher H.J. (1989), 'Cell-polymer interactions: The influence of protein adsorption', Colloids and Surfaces, **42**, 331-343.

Van Wagenen, R.A. and Andrade, J.D. (1980), 'Flat plate streaming potential investigations: Hydrodynamics and electrokinetic equivalency', J. Colloid Interface Sci., **76**, 305-314

van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Schraa, G. and Zehnder, A.J.B. (1987a), 'The role of bacterial cell wall hydrophobicity in adhesion', Appl. Environ. Microbiol., **53**, 1893-1897.

van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Schraa, G.. and Zehnder, A.J.B. (1987b), 'Electrophoretic mobility and hydrophobicity measurements as a measure to predict the initial steps of bacterial adhesion', Appl. Environ. Microbiol., **53**, 1899-1902.

Verrier, D., Mortier, B. and Albagnac G. (1987), 'Initial adhesion of methanogenic bacteria to polymers', Biotechnol. Lett., **9** (10), 735-740.

Wood, J.M. (1980), 'The interaction of micro-organisms with ion exchange resins', in: R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter and B. Vincent (eds.), Microbial adhesion to surfaces, Ellis Horwood Ltd., Chichester, ch. 8, pp. 163-185.

Zobell, C.E. and Allen E.C. (1935), 'The significance of marine bacteria in the fouling of submerged surfaces', J. Bacteriol., **20**, 239-251.

ADHESION OF ANIMAL CELLS ON SURFACES

A. HANDA-CORRIGAN AND R. BRYDGES
School of Biological Sciences
University of Surrey
Guildford, GU2 5XH
England

Introduction

The promotion or prevention of cell adhesion on substrates is important for a vast number of applications in the Biomedical and Biotechnology industries. Materials for biomedical devices include metals (Park, 1984, Katz, 1971) (e.g. stainless steels and Titanium for use in orthopaedic prostheses); native polymers (Polyzois *et al.*, 1991, Tighe, 1992) (e.g. silicone rubber and polyethylene); and polymers which have been surface treated with non-biological (Desai and Hubbell, 1991) or biological molecules such as heparin, urokinase and phosphatidyl choline (Williams, 1992). For any biomedical application the material must firstly and foremostly be non-toxic, non-carcinogenic, non-immunogenic and non-thrombogenic. In addition, a material may be selected for its ability to either promote or prevent cell addition. For example, contact lenses, urinary tract and vascular prostheses are devices which require the minimal of protein adsorption and cell adhesion. On the other hand, for some orthopaedic implants, cell adhesion is encouraged by the use of surface reactive materials such as hydroxyapatite which promotes strong interfacial binding via collagen secreted by the bone cells (Hench and Wilson, 1984).

In animal cell biotechnology, cell-substrate adhesion is required for the growth of anchorage-dependent cells which are exploited for the production of enzymes, vaccines and therapeutic biologicals. Substrates for cell culture include corona-discharged polystyrene, glass beads, microporous carriers of collagen and gelatin, etc. (Chinn *et al.*, 1989, Srivastana *et al.*, 1990). The effective scale-up of anchorage-dependent cell lines requires maximum cell adhesion and growth in:

* Media which are often enriched with highly surface active polymers such as antifoams and cell protecting agents (Pluronic F-68) (Papoutsakis, 1991a).
* High shearing hydrodynamic environments which may prevent cell adhesion, or results in cell detachement and substrate disintegration (Papoutsakis, 1991b).

For both the biomedical and biotechnological applications, a number of complex factors have been shown to affect cell-substrate adhesion. Briefly, these are:

i) Cell type: Adhesion of a specific cell type depends on its morphology (e.g. flat, discoid vs thin, elongated shapes); the density and composition of cell-substrate focal contracts; and the presence of other cell-cell interactions. Spier *et al.* 1987 have demonstrated a significant difference between the strength of adhesion of BHK, Vero and MRC5 cells on cell culture polystyrene.

ii) Substrate wettability: In general cells adhere poorly to hydrophobic substrates. A number of techniques are used to increase the wettability of a substrate: sulphuric acid

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 87–91.

treatment of bacteriological polystyrene (Martin and Rubin, 1974), grafting of poly (vinyl fluoride) with methacrylates (Yoshii and Kaetsu, 1983), plasma discharge of polymeric materials (Amstein and Hartman, 1975), etc.

iii) Surface charge: Both positive and negative surface charges have been shown to affect cell adhesion. Negative surface charges have been demonstrated to be most important on rigid materials such as surface-oxidized polystyrene and glass (Maroudas, 1973), whilst positive charges have been shown to affect cell adhesion on copolymeric hydrogels (Hattori et al., 1985).

iv) Protein adsorption: Although many studies have been carried out on the adsorption of simple proteins onto substrates, little is understood about the effects of the many important cell culture proteins which also contain carbohydrate and sialic acid residues. Protein adsorption onto hydrophobic substrates is often rapid and non-reversible (Kaelbe and Moacanin, 1977), whilst on hydrophilic substrates the interactions are weaker and reversible. It has been suggested however, that proteins with high carbohydrate contents may bind poorly to hydrophobic surfaces (Andrade and Hlady, 1987). Adsorption studies with specific cell adhesion molecules such as fibrorectin show increased cell adhesion in low serum or albumin media, but decreased cell adhesion in media supplemented with high concentrations of non-specific proteins (Grinnell and Feld, 1981).

In conclusion, a number of complex factors affect the adhesion of animal cells onto substrates. In general, most material used for biomedical applications mush have low protein adsorption and cell adhesion, whilst for animal cell biotechnology, materials must promote high cell adhesion and growth. In this study, a simple, reliable method (Radial Laminar Shear Method) has been used to quantify the strength of cell adhesion on a large number of native and surface treated substrates. Contact angle measurements of the substrates have also been determined. The Radial Laminar Shear Method can be used reliably for selecting materials with low, intermediate or high cell adhesion properties.

Materials and Methods

RADIAL LAMINAR SHEAR METHOD FOR DETERMINING THE STRENGTH OF CELL ADHESION TO SUBSTRATES

BHK21 C13 cells cultivated in 50% RPMI/50% DMEM supplemented with 5% newborn calf serum were inoculated onto various substrates at densities of 7×10^4 cells cm^{-2} or 3.9×10^4 cells ml^{-1} for 24 hours at 37'C in 5% CO_2 in air. After 24 hours the cells were subjected to the laminar shear force for 5 seconds in the Cell Detachment Device (CDD). A circular zone of cell detachment was produced on the substrate, the radius of which is known as the critical radius. Four measurements of the critical radius were determined and the mean value was calculated. The critical shear stress required to detach the cells was derived from this equation:

$$To = \frac{3Q_L\eta}{d/_2 H^2} \text{ where}$$

To=surface shear stress (Nm^{-2}), Q_L=flow rate (m^3s^{-1}), η=viscosity ($Kgm^{-1}s^{-1}$), $d/_2$=critical radius (m) and H=gap between the substrate and the CDD (m). Forty sample dishes were tested for each material for 5 seconds at a liquid flow rate of $6.6 \times 10^{-6} m^3\ s^{-1}$.

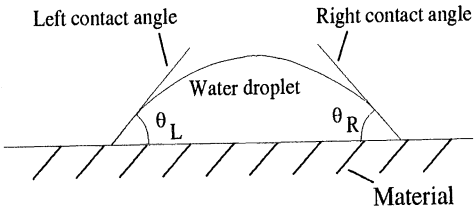


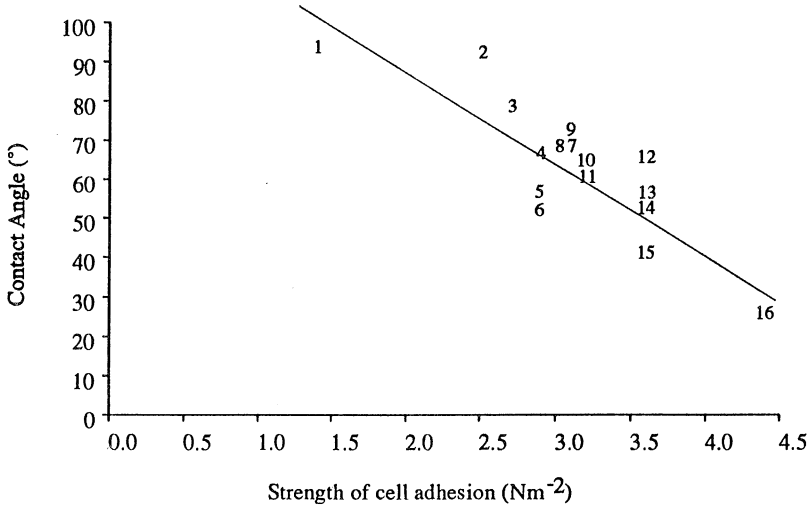
Figure 1 Left and right advancing contact angles on a solid substrate

Substrate wettability was determined by contact angle measurements. These were made on various used, cleaned and sterilised substrates (Brydges, 1991a). A flat sample of the substrate was mounted on a horizontal stage and a 5µl drop of deionised cell culture quality (reverse osmosis and deionised) water (Millipore, USA) was pipetted onto it. This was repeated 10 times. Care was taken not to contaminate the surface of the material at any stage of the measurement. The drop was illuminated from behind, the left (θ_L) and right (θ_R) advancing contact angles were measured with a telescope equipped with a gonimeter eyepiece (see figure 1). The mean values for 10 drops of both the left and right advancing contact angles were used determined. The final measurements for each substrate were therefore evaluated from the mean of 20 results.

Discussion

Cell adhesion and contact angle measurements were carried out on substrates of varying wettability (glasses, plastics, stainless steels and silicone rubber, with and without inert coatings of an impervious hydrocarbon coating called DLC or Diamond-Like Carbon). Figure 2 shows that there is a linear relationship between the strength of cell adhesion (Nm^{-2}) and contact angle values for a number of randomly selected substrates which have been used, cleaned and appropriately sterilised. In a previous publication (Brydges et al., 1991b) contact angles were determined on unused, but cleaned substrates and a linear relationship was not obtained. Some of the materials underwent large changes in contact angles e.g. soda lime microscope glass showed an increase in contact of 74% after being used, cleaned and sterilised. However, some materials, e.g. Silicone rubber and DLC coated polymethylpentene showed small decreases in their contact angles (of 4% and 0.08% respectively). These changes in contact angles therefore explain why we were previously unable to obtain a linear relationship, although, previously we were still able to show that for most materials increasing wettability resulted in increased cell adhesion. In the present paper, we demonstrate that in order to elucidate substrate adhesion properties, it is imperative to determine contact angles on substrates which have undergone the same cleaning and sterilisation procedures as those carried out for the cell adhesion tests.

Substrates which normally support a low strength of cell adhesion (e.g. Polymethylpentene) are able to support a high strength of cell adhesion when they are modified by a coating of DLC. However, the strength of cell adhesion to soda lime microscope glass, a substrate which supports a high strength of cell adhesion was seen to decrease after DLC coating. In addition significant changes in the contact angles were also observed, e.g. Polymethylpentene became more hydrophilic whilst soda lime microscope glass became more hydrophobic. Thus DLC coating of a substrate results in the masking



Material	Strength of cell adhesion (Nm^{-2}) ± standard deviation.	Contact angle(°) ± standard deviation.
1) Polymethylpentene autoclaved.	1.4 ± 1.34	94.00 ± 6.20
2) Silicone rubber autoclaved.	2.5 ± 0.12	94.48 ± 3.45
3) Silicone rubber DLC coated autoclaved.	2.7 ± 0.08	78.88 ± 1.12
4) DLC coated native polystyrene ethanol sterilised.	2.9 ± 0.25	66.94 ± 4.69
5) DLC coated cell culture polystyrene ethanol sterilised.	2.9 ± 0.24	57.02 ± 5.30
6) DLC coated Primaria polystyrene ethanol sterilised.	2.9 ± 0.25	51.20 ± 5.43
7) Stainless steel 316L 2B autoclaved.	3.1 ± 0.25	68.91 ± 2.35
8) DLC coated stainless steel 316L 2B autoclaved.	3.0 ± 0.09	69.04 ± 3.13
9) DLC coated polymethylpentene autoclaved.	3.1 ± 0.25	73.01 ± 2.55
10) DLC coated Stainless steel 316L Bright annealed.	3.2 ± 0.13	64.96 ± 3.86
11) Stainless steel 316L Bright annealed.	3.2 ± 0.10	60.93 ± 4.43
12) DLC coated Alum-borosilicate petri dish glass autoclaved.	3.6 ± 0.40	66.02 ± 3.23
13) DLC coated soda lime microscope slide glass autoclaved.	3.6 ± 0.62	56.43 ± 4.17
14) Alum-borosilicate petri dish glass autoclaved.	3.6 ± 0.56	53.78 ± 4.17
15) DLC coated Primaria polystyrene 1min corona discharge ethanol sterilised.	3.6 ± 0.12	41.26 ± 4.38
16) Soda lime microscope glass autoclaved.	4.4 ± 1.01	26.31 ± 5.72

Fig 2 showing the relationship between strength of adhesion and contact angle on used, cleaned and sterilised materials

of the underlying substrate properties, and the substrate therefore takes on the surface properties of the DLC. DLC can therefore be used as a substrate coating for materials which may not have required properties for cell adhesion.

Conclusion

We have shown that the RLSM can be effectively used for accurately quantifying the strength of cell adhesion on hydrophobic and hydrophilic substrates. This method can be used for selecting biocompatible materials for applications in the Biomedical and Biotechnology areas. We have demonstrated that care must be taken in interpreting the relationships between substrate wettability and cell adhesion. For both the measurements it is advisable to carry out the tests on materials which are cleaned, treated and sterilised as for their final applications in the Biomedical or Biotechnology areas.

References

Amstein, C.F. and Hartman, P. A. (1975) J. Clinical Microbiology, 2, 46-54.
Andrade, J.D. and Hlady, V. (1987) Anal. New York Acad. Sci., 516, 158-172.
Brydges, R. (1991a) Factors affecting the strength of cell adhesion on potentially biocompatible materials, MPhil Transfer Report, University of Surrey.
Brydges, R., Maroudas, N.G. and Handa-Corrigan, A. (1991b) Quantitative measurements of the strength of cell adhesion to substrates of varying wettability, 11th ESACT meeting, Brighton, U.K.
Chinn, J. A., Horbett, T. A., Ratner, B. D., Schway, M. B., Haque, Y., and Hauschka, S.D. (1989) J. Colloid and Interface Sci, 127, 67-87.
Desai, N.P. and Hubbell, J. A. (1991) Biomaterials, 12, 144-153.
Grinnell, F. and Feld, M.K., (1981) J. Biomed. Mater. Res. 15, 361-381.
Hattori, S., Andrade, J.D., Hibbs, J.B., Gregonis, D.E., and King, R.N. (1985) J. Colloid and Interface Sci., 104, 72-78.
Hench, L.L. and Wilson, J. (1984) Nature, 226, 630-636.
Kaelble, D.H., and Moacanin, J., (1977) Polymer, 18, 475-482.
Katz, J.L. (1971) in Biomaterials Bement, A.L. (ed.), University of Washington Press.
Maroudas, N.G. (1973) Nature, 244, 353-355.
Martin, G.R. and Rubin, H. (1974) Exp. Cell Res. 85, 319-333.
Papoutsakis, E.T. (1991a) Tibtech, 9, 316-324.
Papoutsakis, E.T. (1991b) Tibtech, 9, 427-437.
Park, J.B. (1984) in Biomaterials, (ed.) Park, J.B. Plenum Press, New York.
Polyzois, G. L., Winter, R.W., and Stafford, G.D. (1991) Biomaterials, 12, 79-84.
Spier, R.E., Crouch, C. E. and Fowler, H. W. (1987). Develop. Biol. Standard, 66, 255-266.
Srivastana, S., Gorham, S. D. and Courtney, J. M. (1990) Biomaterials, 11, 162-168.
Tighe, B. (1992) in 'Biomedical Technology', Greenshields, R. (ed.), Nordica Printing Company Limited, Hong Kong.
Williams, D. (1992) in Biomedical Technology, Greenshields, R. (ed.), Nordica Printing Company Limited, Hong Kong.
Yoshii and Kaetsu, (1983) Applied Biochemistry and Biotechnology, 8, 505-513.

SHORT-TERM ADHESION STUDIES USING *PSEUDOMONAS FLUORESCENS* CELLS IN CONTACT WITH GLASS AND FLUORINATED ETHYLENE POLYPROPYLENE SURFACES

I.E.C. MOTT AND T.R. BOTT
School of Chemical Engineering
University of Birmingham
Edgbaston
Birmingham B15 2TT. UK

1. Introduction

The deposition and growth of biologically active material, or biofouling, is inherent to most non-sterile aqueous environments. Such a phenomenon is particularly associated with industrial cooling water processes and is a major contributor to operating expenditure.

The morphology and development of biofilms are closely dependent on prevailing system characteristics, for example, fluid velocity and constituents. The surface characteristics of the material of construction will also affect the retention of biological material on the surface. As an alternative, or complementary to the use of biocides, it may be possible to choose a material of construction that is less hospitable to the adhesion of biofilms.

2. Materials and Methods

A rectangular flow unit has been developed to facilitate the study of microbial settling behaviour under controlled flowing conditions (Mott 1987). The objective of the system is to achieve a constant velocity profile across horizontally mounted test sections. The unit construction is required to permit the non-destructive removal of these sections preceding each experiment for staining and microscopic examination.

Perspex was chosen as the material of construction for the apparatus because it may be machined and assembled easily and has a smooth surface with the added advantage of visual assessment. Figure 1 details the overall structure of the closed unit. Sample fluid is passed over three 10 cm diameter glass discs at a constant controlled flowrate by centrifugal pumping. The fluid then collects in a holding tank prior to recirculation. To avoid flow disruption, it is necessary to insert discs flush with the fluid contact surface.

The rectangular flow channel is required to enclose completely the fluid so that no sample microbial aerosols escape to the atmosphere and also ensure the liquid always flows through a constant cross-sectional

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 93–98.

area so that fluid flow characteristics such as Reynolds number may be calculated accurately.

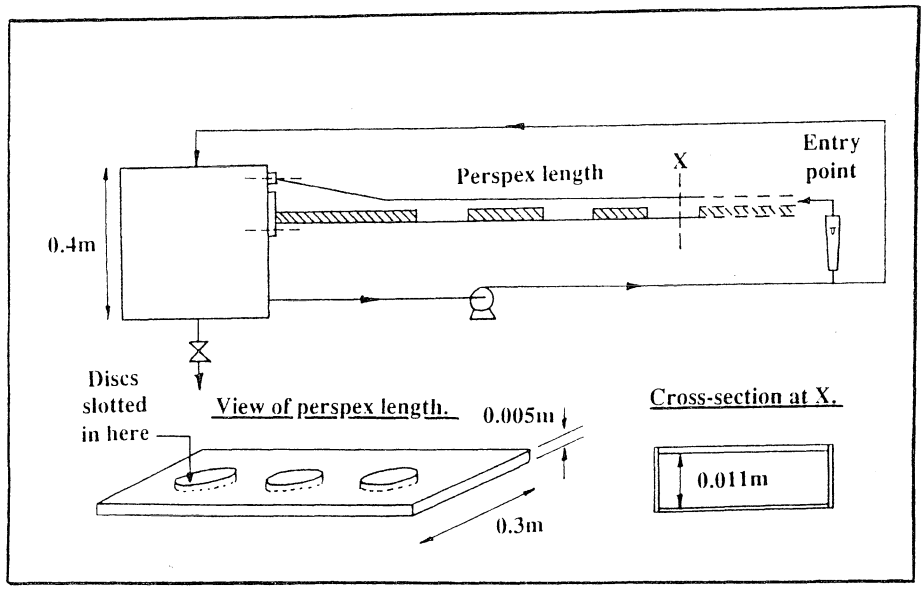


Figure 1. Perspex flow section components

The recirculating fluid consists of a 1 to 2 x 10^7 cells/mℓ *Pseudomonas fluorescens* suspension, dissolved nutrients and mains tap water which has undergone 5 μm and 1 μm pore filtration prior to system entry. *Pseudomonas fluorescens* cells are chosen due to their occurrence and slime forming properties in cooling water systems. The micro-organism is supplied aseptically from a continuously operating fermenter which provides cells within the logarithmic growth phase.

The components of the nutrient solution and to the circulating system are identical, and provides for glucose limiting conditions in the simulated cooling water together with trace elements. The nutrient is identical to that used by Miller (1982).

This paper reports settling and attachment behaviour of *Pseudomonas fluorescens* cells to glass and fluorinated ethylene polypropylene (FEP) test sections. These surfaces are exposed to recirculating sample fluids of mean velocities 0 to 0.41 m/s for periods of 0.5 to 5 hours.

Glass was selected as a control surface during these experiments due to its visual assessment capability, chemical inertness and smooth surface finish. FEP film is manufactured by Holscot Industrial Linings

Ltd. This commercially available product is marketed for high chemical inertness, thermal stability, electrical insulation, transparency and anti-stick properties (Holscot 1988). It may be considered as a potential antifoulant surface due to the latter characteristics.

An FEP film of thickness 0.5 mm is used throughout the investigation. The samples are glued onto glass discs to minimise flow disturbance within the rectangular channel. A 1 cm^2 grid is constructed on the base of all discs to define cell counting boundaries for later microscopic analyses. The discs are then inserted into the perspex apparatus and exposed to the sample fluid under controlled conditions of time and flowrate. After disc removal, the number of cells attached in each grid corner is recorded. The outline of each cell is enhanced by staining with crystal violet prior to microscopic examination. Fifty cell counts are taken per disc and an average taken for each experimental condition.

3. EXPERIMENTAL RESULTS

Results for glass and FEP surfaces are recorded in Tables 2 and 3 and illustrated by Figures 2 and 3. The effect of exposure time and velocity on attachment characteristics of *Pseudomonas fluorescens* cells is demonstrated.

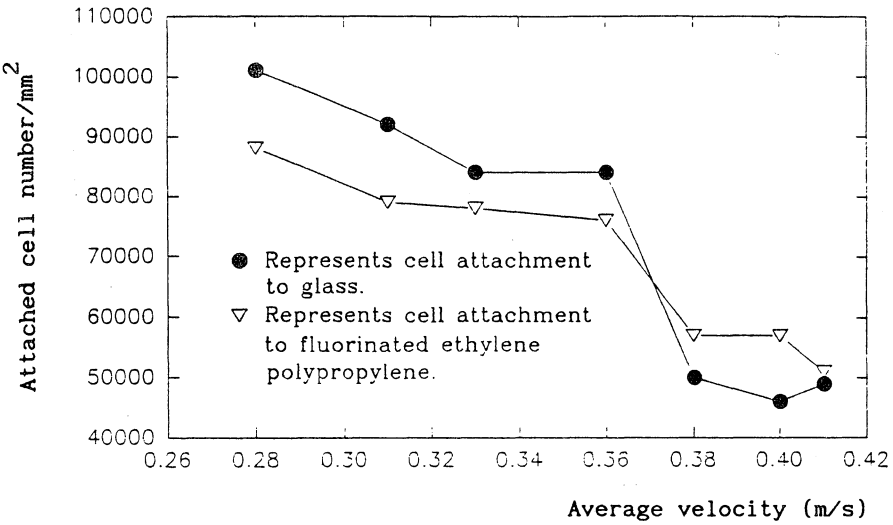


Figure 2. To show the effect of velocity on cell number attachment to glass and FEP surfaces after two hours operation

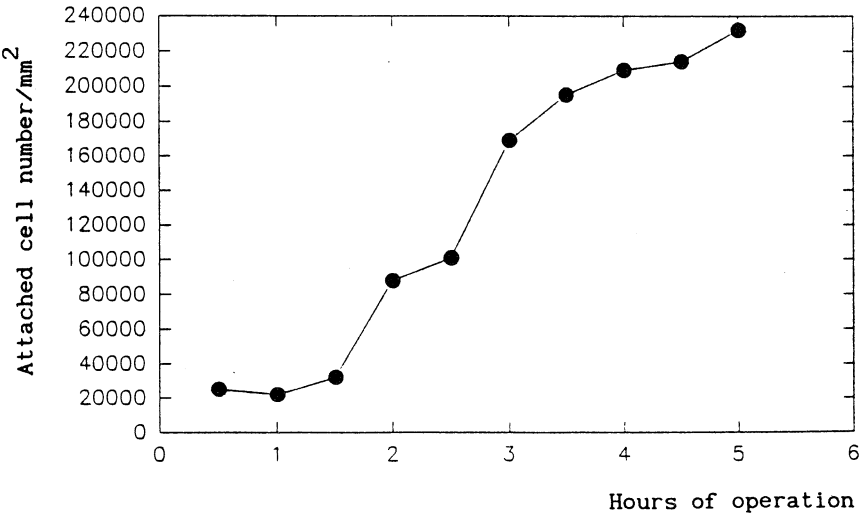


Figure 3. To show the effect of exposure time on cell attachment to glass at 0.38 m/s

4. DISCUSSION

Microscopic examination of all discs subjected to quiescent conditions indicate substantial clumping of cells on the glass and FEP surfaces. Such characteristics are consistent with previous results using glass and novel polymeric surface coatings (Mott 1987).

Conversely, settling experiments under flowing conditions yield a relatively even cell distribution across the entire disc. This is significant because it suggests that constant velocity and fully developed flow conditions are achieved over the entire counting region of the disc. It also indicates that the presence of other adjacent test sections does not appear to influence greatly the attachment characteristics of downstream surfaces. Such factors are essential when a comparison of surfaces and velocities is required.

A comparison of cell settlement characteristics on glass and FEP surfaces is presented by Table 1 and Figure 2. The surfaces are exposed to velocities of 0.28 to 0.41 m/s for 2 hours. The results for both surfaces are similar and indicate a significant correlation between cell number and velocity. A steady decrease of 101000 to 49000 cells/mm^2 for glass and 88000 to 51000 cells/mm^2 for FEP is registered using a

velocity range of 0.28 to 0.41 m/s respectively. The similarity of the surfaces is consistent with other longer-term adhesion data using vertically mounted tubular test sections (Mott 1991). This is encouraging since glass surfaces have consistently exhibited lower fouling levels compared to other materials of construction commonly associated with industrial cooling water systems for example, stainless steel 316 and mild steel (Mott 1991).

TABLE 1. Attached cell number and fluid velocity after 2 hours operation

Test velocity (m/s)	Reynolds number (-)	Attached cell number (cells/mm^2)	
		Glass	FEP
0.00	0	441000	391000
0.28	5880	101000	88000
0.31	6510	92000	79000
0.33	6930	84000	78000
0.36	7560	84000	76000
0.38	7980	50000	57000
0.40	8400	46000	57000
0.41	8610	49000	51000

TABLE 2. The effect of exposure time on cell settling characteristics using a glass surface and a test velocity of 0.38 m/s

Time (hours)	Attached cell number (cells/mm^2)	Time (hours)	Attached cell number (cells/mm^2)
0.5	25000	3.0	169 000
1.0	22000	3.5	195 000
1.5	32000	4.0	209 000
2.0	88000	4.5	214 000
2.5	101000	5.0	232 000

Figure 2 details the effect of exposure time on the settling characteristics of *Pseudomonas fluorescens* cells at 0.38 m/s. A cell concentration of 25000 cells/mm^2 is registered on the glass surface after ½ hour's operation. these initial settlement numbers then appear to remain constant up to 1½ hours operation. an increase of 207000 in attached cell number/mm^2 then prevails up to 5 hours operation, the maximum exposure time examined. It is likely that such an increase may be attributed to the settlement of subsequent cells rather than cell division processes since longer-term studies using this micro-organism

have indicated a significant induction phase of up to 100 hours prior to large-scale cell division (Mott 1991).

5. CONCLUSIONS

A significant correlation between attached cell number and applied velocity is achieved for FEP and glass surfaces. Lower settlement numbers are registered at higher velocities for a test range of 0 to 0.41 m/s.

The length of exposure time influences strongly the extent of attachment on to test surfaces using a velocity of 0.38 m/s. A substantial number of cells (25000 cells/mm^2) are recorded on glass after ½ hour's operation. an increase of 207000 in attached cell number/mm^2 is then observed from 1½ to 5 hours operation, the maximum exposure time.

6. REFERENCES

Holscot (1988). Publicity information, Holscot Industrial Linings Ltd. Grantham, Lincs.

Miller, P.C. (1982). 'Studies in biological fouling, film formation and biocidal removal'. PhD Thesis, University of Birmingham.

Mott, I.E.C. (1987). 'The attachment of *Pseudomonas fluorescens* to specific surfaces'. MSc Thesis, University of Birmingham.

Mott, I.E.C. (1991). 'Biofouling and corrosion studies using simulated cooling water systems'. PhD Thesis, University of Birmingham.

EFFECTS OF SUBSTRATUM HYDROPHOBICITY AND STERIC HINDRANCE ON ADHESION OF A MARINE *PSEUDOMONAS* SP.

K.M. WIENCEK AND MADILYN FLETCHER
Center of Marine Biotechnology
Maryland Biotechnology Institute
University of Maryland System
600 East Lombard Street
Baltimore, Maryland 21202
USA

1. Introduction

Many studies on the adhesion of microorganisms and eucaryotic cells to inanimate surfaces have focused on the chemical and physical properties of the substratum. Several general trends have been observed, such as higher levels of bacterial adhesion and protein adsorption on hydrophobic surfaces as compared to hydrophilic substrata (Marshall, 1985; Pringle and Fletcher, 1983). However, there is enough conflicting evidence to prevent the prediction of bacterial adhesion based solely on substratum hydrophobicity (Dexter *et al.*, 1975; Pedersen *et al.*, 1986). Steric repulsion may retard adhesion of proteins (Lee, *et al.*, 1988; Prime and Whitesides, 1991), eucaryotic cells (Owens *et al.*, 1987), and bacteria (Humphries *et al.*, 1987) to surfaces. Molecular mobility of flexible molecules on the substratum surface sterically hinders the adhesion of bacteria, regardless of the chemical composition of cell surface adhesives. To investigate the effects of substratum molecular mobility and chemical composition on the kinetics and strength of adhesion, we have initiated studies using substrata which exhibit a homogenous surface consisting of specific functional groups.

2. Methods

2.1. ORGANISM

Cell cultures of marine *Pseudomonas* sp. NCIMB 2021 were grown to stationary phase in PYE medium (0.1% peptone, 0.07% yeast extract, 3.3% Instant Ocean (Aquarium Systems, Mentor, Ohio)) at 20C°. Cultures were harvested by centrifugation, washed twice, and resuspended in 3.3% Instant Ocean (w/v in distilled water) to a density of 2.0-5.0 x 10^8 cells/ml.

2.2. SUBSTRATA

Substrata with different surface characteristics were prepared in two ways, as follows: (1) Glass coverslips were cleaned in 4:1 H_2SO_4/30% H_2O_2. Glass was rendered hydrophobic (advancing

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 99–104.

contact angle (Θ_a)(water)= 90°) by immersion in a commercial silane, *SigmaCote* (Sigma Chemical Co., St. Louis, MO). (2) Three types of self-assembled monolayers (SAMs) composed of long-chain (10-18 carbons) alkanethiols were applied to glass coverslips. Decanethiol and octadecanethiol were applied individually or as a mixture to construct SAMs based on the methods of Bain and Whitesides (1989) and Bain *et al.* (1989). Clean glass coverslips were coated with a thin (20 nm) layer of gold metal. To facilitate firm adsorption of the gold, (3-mercaptopropyl)trimethoxysilane was applied to the coverslips prior to deposition of gold metal (Goss *et al.*, 1991). The monolayers were assembled onto the gold by immersion of the gold-coated coverslips into an ethanol solution of the alkanethiol. The sulfur moieties of the alkanethiol bind to the gold, causing the molecules to pack tightly in an orientation nearly perpendicular to the surface of the gold. The resulting monolayer forms a nearly crystalline structure within the long-chain alkane region. The surface characteristics of SAMs are dependent on chain length and the terminal functional group (or groups for a mixed monolayer), which can consist of methyl, hydroxyl, carboxyl, or oligo(ethylene glycol) moieties (Bain *et al.*, 1989; Prime and Whitesides, 1991). We have constructed SAMs composed of decanethiol, octadecanethiol, and a mixture of both alkanethiols. The single-component monolayers of decanethiol and octadecanethiol exhibited the tightly-packed crystalline structure described above, with methyl groups exposed at the surface. Advancing contact angles of water and hexadecane confirmed that the single component methyl-terminated monolayers exhibited a very hydrophobic surface (Θ_a(water)=98°; Θ_a(hexadecane)=32°). The mixed-component monolayer (decanethiol/octadecanethiol, 15:1 in solution) was composed of octadecanethiol chains extending above a tightly-packed mixture of decanethiol and octadecanethiol. The mixed-component monolayer exhibited hydrophobic characteristics with water, a polar liquid. However, hexadecane spread readily on the exposed alkyl chains of octadecanethiol, reportedly due to the mobile or fluid-like state created by the exposed chains of the longer alkanethiol (Bain and Whitesides, 1989).

2.3. FLOW CHAMBER

Two types of laminar flow chamber were employed: rectangular glass capillary tubes (0.4 mm x 4 mm x 100 mm, Vitro Dynamics, Inc., Rockaway, NJ) and a polycarbonate chamber (0.5 mm x 14 mm x 25 mm) similar to the design of Owens *et al.* (1988). Laminar flow was confirmed by dye tests conducted in the employed range of volumetric flow rates.

2.4. MICROSCOPY AND IMAGE ANALYSIS

Two microscope techniques were used in conjunction with computer-controlled image analysis. The kinetics of adhesion (numbers of bacteria attaching and detaching per unit time) to several transparent substrata were measured with phase-contrast microscopy (Axioplan, Zeiss, Thornwood, NY). Reflection contrast microscopy (RCM) with an antiflex objective was used to evaluate the separation distance between an attached cell and a glass or silane-coated glass coverslip substratum. With this technique, image intensity is a function of separation distance, so that at minimum separation distance, an image of maximum darkness is obtained. As separation distance increases, the image becomes increasingly bright up to a distance of approximately 100 nm (Verschueren, 1985). Attached cells, including total area covered, the percentage of cells which attached or detached since the previous measurement, and the average orientation of cells with respect to the axis of flow, were measured (*ca.* 2 min intervals) with computer programmed image analysis (IBAS 2000, Kontron, Eching, Germany). Images of

attached cells obtained from RCM were captured and saved for later analysis.

3. Results

3.1. ADHESION KINETICS

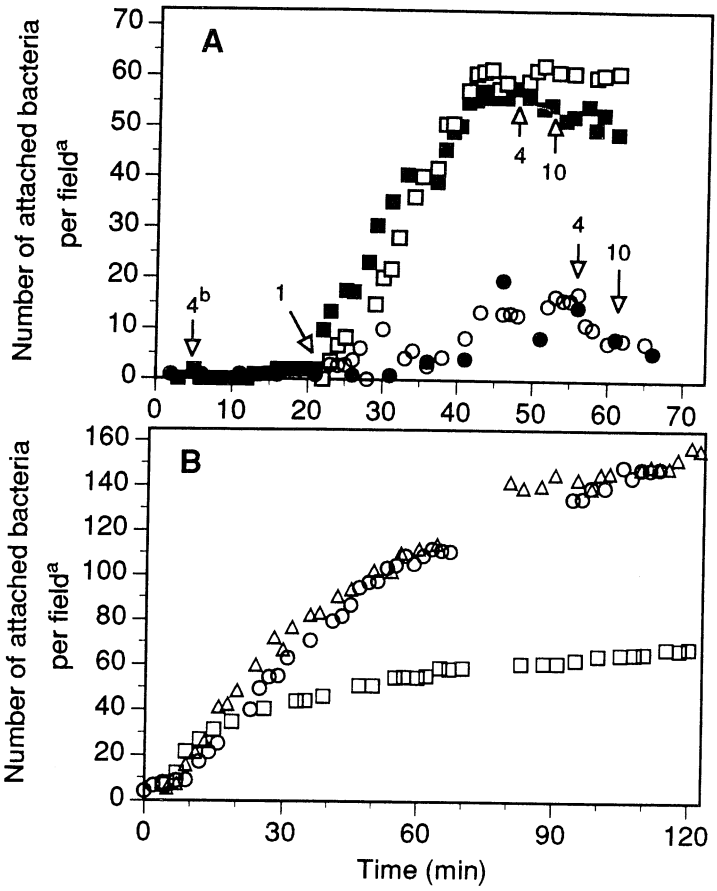


Figure 1. Kinetics of adhesion of marine *Pseudomonas* sp. NCIMB 2021 to inanimate substrata under laminar flow. (A) Effects of flow rate. Arrows designate changes in flow rate (ml/min). Glass (●,○) and silane-coated glass (■,□). Two representative trials are illustrated for each substratum. (B) SAMs of decanethiol (□), octadecanethiol (○), and a 15:1 mixture of both alkanethiols (△) under a flow rate of 1 ml/min. Representative trials are illustrated for each surface.

[a]field=4577 μm^2

[b]volumetric flow rates (ml/min)

The kinetics of adhesion of marine *Pseudomonas* sp. NCIMB 2021 to glass and to hydrophobic, silane-coated glass was measured in glass capillary tubes. Cells adhered much faster and to a greater extent to the hydrophobic coating than to uncoated glass (Figure 1A). During the first 20 min of exposure, adhesion to either substratum did not occur at a volumetric flow rate greater than 1 ml/min (fluid velocity = 1 cm/s). A flow rate of 5 ml/min (5 cm/s) was sufficient to remove most cells from the glass, but had little effect on the cells attached to the hydrophobic coating. A 10 ml/min flow rate removed some cells from the silane-coated glass, but results were not reproducible. The number of cells attaching to each substratum over time also differed in the degree of scattering of data within each trial (Fig. 1A). Opposed to the smooth, consistent kinetics profile of cells attached to hydrophobic glass, the number of cells attached to glass fluctuated throughout the 65 min exposure period, regardless of flow rate. Image analysis also indicated that cells detached from glass much more readily than they did from the hydrophobic substratum.

The kinetics and extent of adhesion to three types of SAMs was measured in a polycarbonate laminar flow cell at a fixed flow rate of 1 ml/min (0.24 cm/s). Although the rates of adhesion over the first 15 min of exposure were similar for the three hydrophobic surfaces, there were notable changes in the area covered after two hours exposure (Figure 1B). Attachment of cells to decanethiol SAMs did not increase after *ca.* 20 min exposure, whereas cells continued to attach to the octadecanethiol and mixed-component SAMs for up to 90 min of exposure. Adhesion to the mixed-component monolayer was slightly higher than to the single component octadecanethiol monolayer. The percentage of cells which attached or detached between successive time periods was similar for the three hydrophobic surfaces.

3.2. REFLECTION CONTRAST MICROSCOPY (RCM)

RCM-generated images of cells attached to glass or hydrophobic glass were enhanced and analyzed. Preliminary results indicated that the dark interference pattern (consistent with minimum separation distance) occurred in many of the cells attached to hydrophobic glass but not uncoated glass (data not shown). Among the cells attached to hydrophobic glass, the size of the interference pattern varied, but usually occurred at one or both ends of the cell.

4. Discussion

With real-time image analysis of bacterial adhesion kinetics, we were able to quantitatively determine the kinetics of attachment and detachment from the substratum over a two hour exposure period. The relatively low levels of detachment from the hydrophobic surface may be related to the strength or type of adhesion mechanism. Adhesion to a hydrophilic surface such as glass appeared to involve weaker interactions than adhesion to hydrophobic substrata, as evidenced by the relatively slow flow rates required to remove attached from glass as compared to silane-treated glass. The increased amount of cell attachment and detachment from glass at a constant flow rate (compared to treated glass) also indicated weak interactions between the bacterial surface and glass. RCM analysis of the marine pseudomonad attached to glass indicated an increased separation distance between the bacteria and the hydrophilic surface, compared to the hydrophobic silane coating. Most cells attached to the hydrophobic substrata did not exhibit the Brownian motion associated with "reversible adhesion" (van Loosdrecht *et al.*, 1989), whereas most cells attached to glass continued to exhibit Brownian motion even after two hours of attachment. The observed increase in the rate of adhesion to a relatively hydrophobic substratum

has been reported (Marshall, 1985; Rosenberg, 1986; Vanhaecke *et al.*, 1990). The observed effects of volumetric flow rate and shear stress on adhesion and removal of attached cells from hydrophobic and hydrophilic substrata also has been documented (Owens *et al.*, 1987; van Loosdrecht, 1989).

Self-assembled monolayers (SAMs) were synthesized from two long-chain alkanethiols, i.e. decanethiol and octadecanethiol. The number of cells that attached to the two-component SAM was substantially higher than attached numbers on the decanethiol surface and slightly higher than attachment to octadecanethiol. These initial data indicate that the molecular mobility of the exposed alkyl chains of octadecanethiol on the mixed SAM do not prevent adhesion of the pseudomonad. The higher numbers of cells attached to the mixed monolayer may be due to increased interaction by means of dispersion forces on this substratum, as evidenced by decreased contact angles of hexadecane. Currently, we are unable to adequately explain the relatively low amount of cell adhesion to the decanethiol SAM compared to the octadecanethiol SAM. It has been reported that alkanethiol SAMs with alkyl chains of less than ten carbons may form monolayers which allow exposure of the underlying gold surface (Bain *et al.*, 1989). It is possible that the ten carbon chain of decanethiol is not long enough to prevent the influence of the gold metal on bacterial adhesion, but the effects of gold were not detected by contact angle measurements. To ensure that interactions with the gold metal are masked, future studies will include the eleven carbon alkanethiol, undecanethiol. Also, intact monolayers will be verified by ESCA analysis.

The methodologies described here will provide the basis for a detailed study of the chemical and physical effects of substratum composition on several aspects of bacterial adhesion, including adhesion kinetics, strength of adhesion, and the separation distance between attached bacteria and the substratum. Using the image analysis system to simultaneously measure attached cells of morphologically different bacterial species, we are initiating studies on competition among bacteria for an exposed substratum. Other planned studies include the synthesis of mobile and immobile hydrophilic alkanethiol monolayers, and the addition of different silane coatings for a detailed analysis of bacterial adhesion with reflection contrast microscopy.

5. Acknowledgements

This work was supported in part by Courtaulds Coatings Ltd., UK. K.M.W. thanks the National Science Foundation for a travel fellowship.

References

Bain, C. D., Troughton, E. B., Tao, Y., Evall, J., Whitesides, G. M., and Nuzzo, R. G. (1989) "Formation of monolayer films by the spontaneous assembly of organic thiols from solution onto gold", J. Am. Chem. Soc. 111:321-335.

Bain, C. D., and Whitesides, G. M. (1989) "Formation of monolayers by coadsorption of thiols on gold: variation in the length of the alkyl chain", J. Am. Chem. Soc. 111:7164-7175.

Dexter, S. C., Sullivan, J. D., Williams III, J., and Watson, S. W. (1975) "Influence of substrate wettability on the attachment of marine bacteria to various surfaces", Appl. Microbiol. 30:298-308.

Goss, C. A., Charych, D. B., and Majda, M. (1991) "Application of (3-mercaptopropyl)trimethoxysilane as a molecular adhesive in the fabrication of vapor-deposited gold electrodes on glass substrates", Anal. Chem. 63:85-88.

Humphries, M., Nemcek, J., Cantwell, J. B., and Gerrard, J. J. (1987) "The use of graft copolymers to inhibit the adhesion of bacteria to solid surfaces", FEMS Microbiol. Ecol. 45:297-304.

Lee, J. H., Kopecek, J., and Andrade, J. D. (1989) "Protein-resistant surfaces prepared by PEO-containing block copolymer surfactants", J. Biomed. Mater. Res. 23:351-368.

Marshall, K. C. (1985) "Mechanisms of bacterial adhesion at solid-water interfaces" in D. C. Savage and M. Fletcher, (eds.), Bacterial Adhesion. Plenum Press, New York, KG, Berlin, pp. 133-156.

Owens, N. F., Gingell, D., and Rutter, P. R. (1987) "Inhibition of cell adhesion by a synthetic polymer adsorbed to glass shown under defined hydrodynamic stress", J. of Cell Sci. 87:667-675.

Owens, N. F., Gingell, D., and Trommler, A. (1988) "Cell adhesion to hydroxyl groups of a monolayer film", J. of Cell Sci. 91:269-279.

Pedersen, K., Holmstrom, C., Olsson, A., and Pedersen, A. (1986) "Statistic evaluation of the influence of species variation, culture conditions, surface wettability and fluid shear on attachment and biofilm development of marine bacteria", Arch. Microbiol. 145:1-8.

Prime, K., and Whitesides, G. M. (1991) "Self-assembled organic monolayers: model systems for studying adsorption of proteins at surfaces", Science 2522:1164-1167.

Pringle, J. H., and Fletcher, M. (1983) "Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces", Appl. Environ. Microbiol. 45:811-817.

Rosenberg, M., and Kjelleberg, S. (1986) "Hydrophobic interactions: role in bacterial adhesion", Adv. Microbiol. Ecol. 9:353-393.

Vanhaecke, E., Remon, J., Moors, M., Raes, F., De Rudder,D., and Peteghem, A. V. (1990) Kinetics of *Pseudomonas aeruginosa* adhesion to 304 and 316-L stainless steel: Role of cell surface hydrophobicity" Appl. Environ. Microbiol. 56:788-795.

van Loosdrecht, M. C. M., Lyklema, J., Norde, W., and Zehnder, A. J. B. (1989) "Bacterial adhesion: a physicochemical approach", Microb. Ecol. 17:1-15.

Verschueren, H. (1985) "Interference reflection microscopy in cell biology: methodology and applications", J. Cell Sci. 75:79-301.

COATED SURFACES IN RELATION TO BIOFILM FORMATION

R. SANTOS and T.R. BOTT
School of Chemical Engineering
University of Birmingham
Edgbaston
Birmingham B15 2TT. UK

M.E. CALLOW
School of Biological Sciences
University of Birmingham
Edgbaston
Birmingham B15 2TT. UK

1. Introduction

The use of coated surfaces could be an alternative to the use of biocides for the control of biofilms.

In order to establish the effects of certain variables such as flow velocity on treated surfaces a special apparatus has been developed in which biofilms of *Pseudomonas fluorescens* may be grown under controlled conditions. *P. fluorescens* was chosen for the work because it is known to be an early coloniser of surfaces in mixed systems and occurs in natural environments, eg. in rivers, lakes and soils. *P. fluorescens* is one of the most common slime forming micro-organisms found in cooling water systems (Liebert and Hood, 1985). A single species was chosen to illustrate the effects of changes in the variables in order to facilitate the subsequent analysis of the data. Furthermore, to obtain steady state conditions, experience has shown that a culture of single species is easier to control than a mixed culture simulating an industrial water that may contain a large number of different organisms.

2. Materials and Methods

The principle of the operation is shown on Figure 1. The apparatus is a more elaborate version of the one previously used in biofouling research (Bott and Miller, 1983), and the experimental conditions are generally similar.

The flow of cell suspension from the fermenter to the mixing vessel was arranged to give 2×20^7 cells.$m\ell^{-1}$ in the circulating water and the flow of medium to provide 8 $mg\ell^{-1}$ glucose at the beginning of each experiment.

Ferric citrate (2.7 $g.\ell^{-1}$ solution) was added to the mixing vessel at a flow rate of 0.01 $m\ell.s^{-1}$, in addition to the nutrient solution in order to make up the iron deficiency resulting from the use of distilled water.

Conditions in the mixing vessel in respect of pH, temperature and

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 105–110.

dissolved oxygen were carefully monitored. The pH was maintained at 7.0 by continuous addition of 0.5 M KOH. The mixing vessel was "sparged" with filtered air.

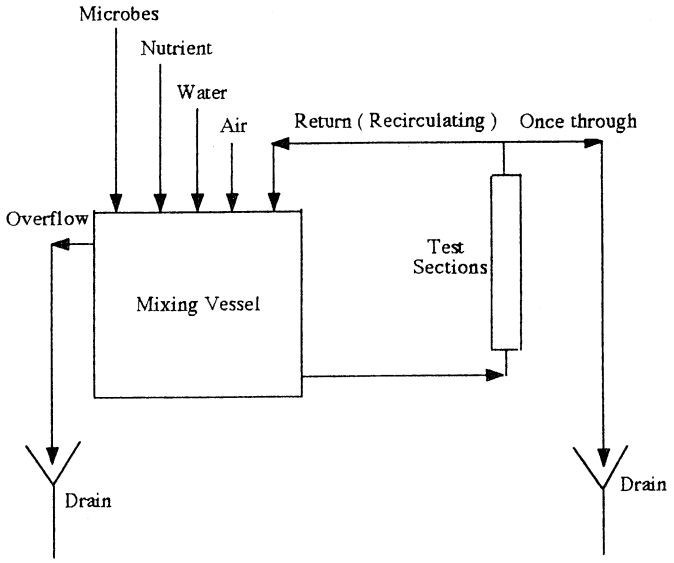


Figure 1. Generalised drawing of the simulated cooling water system

Seven test sections consisted of glass tubes 767 mm long and either 16.5 or 11.0 mm internal diameter, giving velocities of 0.5 or 2.5 ms^{-1} respectively, ie. Reynolds number of 9667 and 32222 respectively. Each tube had its own rotameter so that the flow in each tube could be carefully controlled. Assessment of biological growth *in situ* on glass test sections was made using an infra red device. The principle of the measurement is based on the absorbance of infra red radiation as it passes through the biofilm.

It was recognised that glass is not a normal material of construction for heat exchangers, but the "non-stick" properties of the polymers could be effectively investigated by the technique provided that a suitable "anchor" to the glass surface could be established. Furthermore a glass surface is generally not hospitable to biofilm adhesion, so the comparison of the polymer treatment with a glass control represents a relatively severe test. Before tests could be carried out in the flow equipment it was necessary to develop suitable techniques for coating the tube surfaces. The criterion was essentially to give uniform thin layers free from surface cracks and irregularities.

A sample of the recirculating fluid was taken for microbiological tests to ensure that there was no contamination prior to the addition of the stream containing the *P. fluorescens*.

Analysis of glucose concentration was carried out daily during an

experiment. The glucose concentration was observed to respond to the addition of the *P. fluorescens* and fall to below 1 mgℓ^{-1} accompanied the initial colonisation of the test surface. The temperature of the fouling fluid was kept as close as possible to 27°C throughout the experiment.

3. Results and Discussion

Infra red absorbance of biofilm growth at velocities of 0.5 and 2.5 ms^{-1} on glass test sections when distilled water is used as the make-up water, shows that at lower velocities the biofilm grows more rapidly and thicker than at high velocities. The effects of fluid velocity are clearly demonstrated, with a thicker film being produced at a velocity of 0.5 ms^{-1} compared to 2.5 ms^{-1}. The thinner film at higher velocity is attributed to the effects of increased fluid shear at 2.5 ms^{-1} compared to that a low velocities. As demonstrated by Santos *et al* (1991), the less stable biofilm produced at 0.5 ms^{-1} suggested a biofilm with a less robust character and visual observation revealed that the biofilm grown at lower velocity was much more open and "fluffy" than that grown at 2.5 ms^{-1} which appeared much more compact.

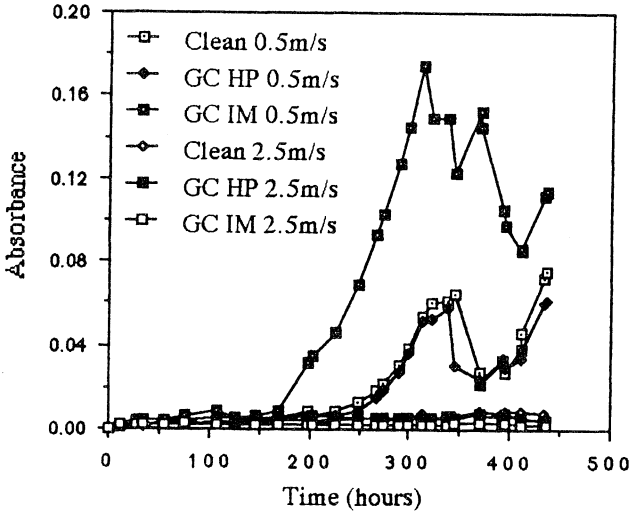


Figure 2. Polymers compared with control at 0.5 and 2.5 ms^{-1} using distilled water

The effect on biofilm growth of glass coated with two different chemical modifications which did not alter the absorbance properties of the glass is shown in Figure 2. The surface treatments used were Glassclad HP (GC HP) and Glassclad IM (GC IM) from Fluorochem Ltd. Glassclad HP is a heparin modified siloxane resin providing hydrophilic

surfaces with low thrombogenicity. Glassclad IM is a polyethyleneimine modified resin used to provide glass surfaces with greater affinity for cell adhesion.

As shown in Figure 3, the biofilm develops faster and becomes thicker on a Glassclad IM coated surface than on either the control glass or Glassclad HP coated surface at 0.5 ms^{-1} velocity. There is no difference in the biofilm growth at 2.5 ms^{-1} velocities for the three surfaces. Examination of the biofilms by SEM reveals that at higher velocity the biofilm is more compact and the cells are aligned in the direction of the flow (Santos *et al*, 1991).

There are no major differences in the biofilm growth on the control glass and Glassclad HP test section which was to be expected, since the surface properties of both were similar.

All surfaces exhibit the typical sloughing and regrowth pattern as described by Bott and Miller (1983).

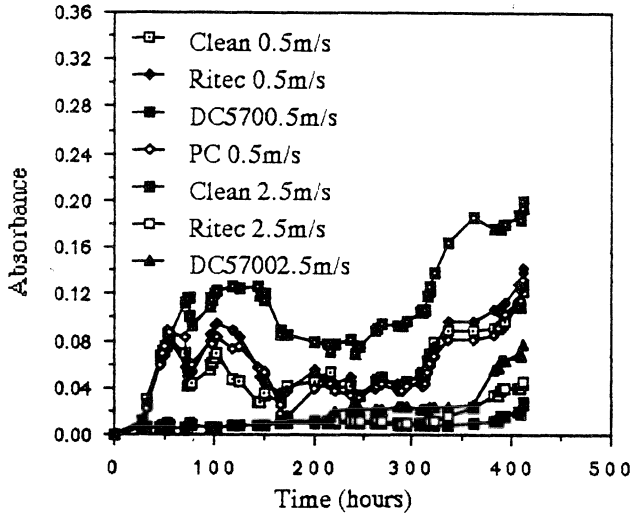


Figure 3. Polymers compared with control at 0.5 and 2.5 ms^{-1} using distilled water

The pattern of infra red absorbance of biofilm growth at lower velocities being greater than at higher ones was observed throughout all the experiments, when distilled water is used as the make-up water. The biofilms produced were composed exclusively of *P. fluorescens*. A detailed description of biofilms using distilled water as the make-up water is given in Santos *et al* (1991).

As shown in Figure 3, the performance of the polymers in preventing biofilm adhesion when compared with the control (untreated surface) follow the same pattern at both velocities, where the biofilm growth is greater in the test sections coated with DC 5700.

Both Ritec Clearshield and Perma Clear are silane coupling agents with

characteristics very similar to glass. This is in agreement with the results shown in Figure 4, where there is not much difference in the biofilm growth between the test sections coated with Ritec and Perma Clear, and the control one.

Ritec Clearshield is a trimethyl chloro silane or trimethyl ethoxy silane. Perma Clear consists of an alcoholic solution of siloxane polymer.

Dow Corning 5700 antimicrobial agent (DC 5700) is trimethoxysilyl propyloctadecyidimethyl ammonium chloride diluted to 42% active ingredients by weight with methanol.

In this experiment, the tap water was used as the make-up water instead of the distilled water. It passed sequentially through a 5μm pore activated charcoal cartridge filter to remove chlorine, a 1 μm cartridge filter to remove all living organisms and spores.

When filtered tap water was used the biofilm growth at 2.5 ms^{-1} was much thicker compared with the biofilm growth at 0.5 ms^{-1}. The structure of the biofilms were very different both between the different sources of the make-up water and between the two velocities using any make-up water. Another relevant factor was the remarkable difference in the colour of the biofilm when distilled water was used (pale-white colour biofilm) compared with the biofilm when filtered tap water was used (orange-yellow colour biofilm). In order to try to explain these differences, identification tests were carried on the biofilm at the end of the experiment by scraping some of the biofilm from the control test sections and sent for analysis. The results show that no *Pseudomonas fluorescens* was present in the biofilm instead *Pseudomonas paucimobilis* (pigmentation yellow) together with *Chryceomonas luteola* (pigmentation orange) were present.

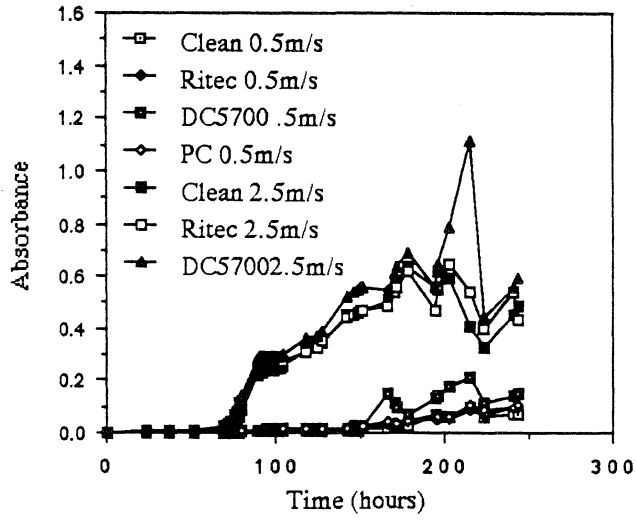


Figure 4. Polymers compared with control at 0.5 and 2.5 ms^{-1} using filtered tap water

It is assumed that the filter system was inadequate in the experiments with tap water so that contamination could occur. The only positive identification for *Pseudomonas fluorescens* was found in the samples from the fermenter and from the biofilm grown when distilled water was used.

The test results in relation to the performance of the surface coatings were similar for the two types of water,. The results however, were surprisingly different, if to not say, the opposite in respect of adhesion at the lower and the higher flow velocities. This is shown in Figure 4 where the infra red absorbance of the biofilms at 2.5 ms^{-1} are much greater than at 0.5 ms^{-1}. When compared with the results shown in Figure 3 in respect of velocities, they are completely different. For example, in Figure 5 where filtered tap water was used, at higher velocity rapid biofilm growth was seen after 80 hours, growth continuing up to 178 hours with absorbance values of approximately 0.6, when the first sloughing occurred.

The pattern of biofilm deposition and growth is very different when distilled water is used (Figure 3). At the higher velocity 2.5 ms^{-1} only a thin biofilm develops and at 200 hours corresponding to an absorbance value of 0.09 is only approximately 15% of that seen after 200 hours at the same velocity with filtered tap water as the diluent. For the lower velocity 0.5 ms^{-1}, although the biofilm starts to grow after 32 hours, after 200 hours (prior to when two sloughing had occurred) the absorbance value of 0.04 is approximately 50% of that seen after 200 hours at the same velocity with filtered tap water as make-up water for the control, Ritec and Perma Clear, but for the Dow Corning 5700 at the same velocity and hours run the absorbance is approximately 80%.

4. Acknowledgements

The authors wish to express their thanks to Mr. Geoff Bartlett from the Electronic Workshop of the School of Chemical Engineering, University of Birmingham, who developed the infra red biofilm monitoring device. Thanks are also due to SERC and International Paint Ltd. for the financial support.

5. References

Bott, T.R. and Miller, P.C. (1983). *Mechanisms of biofilm formation on aluminium tubes.* J. Chem. Technol. Biotechnol., 33B: 177-184.

Christensen, B.E., Tronnes, H.N., Vollan, K., Smidrod, O. and Bakke, R. (1990). *Biofilm removed by low concentration of hydrogen peroxide.* Biofouling 2: 165-175.

Liebert, C. and Hood, M.A. (1985). *Characterisation of bacterial population in an industrial cooling system.* Dev. Ind. Microbiol. 26: 649-660.

Santos, R., Callow, M.E. and Bott, T.R. (1991). *The structure of Pseudomonas fluorescens biofilms in contact with flowing systems.* Biofouling 4: 319-336.

Chapter 3

METABOLISM

BACTERIAL METABOLISM IN BIOFILMS

MADILYN FLETCHER
Center of Marine Biotechnology
Maryland Biotechnology Institute
University of Maryland System
600 E. Lombard St.
Baltimore, Maryland 21202
USA

1. Introduction

Bacteria that are attached to surfaces frequently appear to differ metabolically from their free-living counterparts. There are two probable reasons for such physiological differences: (1) the physicochemical conditions at the solid surface-liquid interface are not the same as those in the bulk phase, and thus influence bacterial metabolism through environmental effects, and (2) the bacteria in biofilms are often situated in close proximity to other bacteria, microorganisms, and often macroorganisms, and are influenced by synergistic, mutualistic, competitive, or antagonistic interactions among biofilm members.

Numerous studies have been conducted to determine the ways in which solid surfaces influence bacterial activity, and attempts have been made to identify those principles that explain the basis of surface effects. Some general observations may be made. For example, bacteria in biofilms tend to be less susceptible to toxic substances than freely suspended cells. However, for the most part, it has been impossible to formulate any general principles that describe the influence of surfaces on bacterial physiology, much less determine the mechanistic basis for such effects. This is because of the great variability and diversity of bacteria, with respect to taxonomic, functional, and genetic characteristics. The relative significance of influential factors at the solid-liquid interface will depend not only upon the physiological capabilities of the organisms, but also on ambient nutrient conditions, and other physicochemical factors, such as oxygen tension or electrolyte concentration, the nature of the solid surface, and water flow and turbulence. All of these elements contribute to the development of a biofilm community and help to determine its composition and physiological activity.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 113–124.

2. Conditions at the Surface

2.1. NUTRIENTS AND SURFACES

The two most important characteristics of the solid-liquid interface for bacterial metabolism are (a) hydrodynamic conditions, which are different from those in the bulk phase, and (b) the tendency for dissolved solutes and particles to be adsorbed at the surface. These two factors affect the concentration and flux of nutrients, as well as removal of metabolic products, at the bacterial biofilm. In flowing systems, mass transfer of nutrients to the biofilm will tend to increase with flow velocity (Characklis, 1984). Also, for rough surfaces, including those with biofilms, mass transfer is increased, possibly as much as three-fold (Characklis and Marshall, 1990, p. 326), compared to smooth surfaces. Thus, in general, bacteria in biofilms in flowing systems are at an advantage because of increased delivery of nutrients and removal of inhibitory metabolites.

There is also a tendency for dissolved solutes to be adsorbed on surfaces, because the accompanying decrease in interfacial free energy is favored thermodynamically (Hoffman, 1986; Norde, 1986). Accordingly, the adsorption of nutrients at surfaces may concentrate them and make them more accessible to attached bacteria. This is particularly true for high molecular weight compounds, e.g., proteins, carbohydrate polymers, which would have multiple binding sites and, thus, tend to be retained at the surface. Such macromolecules must be hydrolyzed before assimilation by the bacteria. Thus, surface immobilization may promote access of bacterial enzymes to the substrates, and thus facilitate utilization by bacteria. This process could be of prime importance in many natural environments where readily assimilated low-molecular-weight substrates (e.g., amino acids, sugars, organic acids) are scarce.

Although adsorption of macromolecular nutrients on surfaces could theoretically increase their availability to bacteria, it can, in some circumstances, have the opposite effect. Avid binding of substrates could prevent their hydrolysis by bacteria, or adsorption of bacterial enzymes could inhibit or reduce their activity (Estermann et al., 1959; Quiquampoix, 1987; Quiquampoix et al., 1989). Nucleic acids have been found to be protected against degradation through interactions with sediment or sand fractions (Lorenz et al., 1981; Lorenz and Wackernagel, 1987). Similarly, bacterial breakdown of complex organic compounds, such as 2,4-D ([2,4-dichlorophenoxy] acetic acid)(Ogram et al., 1985) or diquat (6,7-dihydrodipyrido[1,2-*a*:2',1'-*c*]-pyrazidinium dibromide) (Weber and Coble, 1968) can be inhibited through adsorption on particles.

Solid surfaces may also be substrates, in which case they offer a clear advantage for attached bacteria. Bacteria that utilize cellulose (Lamed et al., 1987), in particular *Clostridium thermocellum* (Bayer et al., 1985), have evolved surface-associated structures (e.g., polycellulosomes) that contain exocellular cellulase and mediate contact with cellulose surfaces. Also, some bacteria appear to degrade solid substrates, such as wood (Holt and Jones, 1983) or steroid crystals (Zyvagintseva and Zvyagintsev, 1969), in the near proximity of the cell, so that "craters" are produced in the substrate beneath the organisms. In some cases, the oxidation or reduction of minerals, e.g., sulfur

(Takakuwa et al., 1979), iron oxides (Munch and Ottow, 1982), by bacteria has required contact between mineral and bacterial surfaces. Frequently, such solids that serve as substrates, e.g., cellulose, lignin, are largely recalcitrant, and represent the residual that is left after leaching out and assimilation of more easily utilized substrates, e.g., amino acids, sugars, organic acids.

2.2. THE BIOFILM AS A MICROENVIRONMENT

Solid surfaces have different environmental characteristics from aqueous systems, not only because of the differences in physicochemical factors, but also because of the unique environment that is created through the development of the biofilm itself. The adsorption of macromolecular compounds and attachment of individual bacteria are only the first stages in biofilm development. This is followed by the growth of bacteria (if nutrients are sufficient), development of microcolonies, recruitment of additional attaching bacteria, and often colonization by other organisms, e.g., microalgae, macroalgae, invertebrates. As the bacteria grow, extracellular polymers are produced and accumulated, so that the bacteria are eventually embedded in a highly hydrated polymeric matrix. This polymeric material is largely polysaccharide, and, to a certain extent, acts as a gel diffusion barrier (Costerton et al., 1985). The biofilm bacteria are consequently immobilized and, thus, dependent upon substrate flux from the adjacent liquid phase and/or exchange of nutrients with their neighbors in the biofilm.

Little is known about the way in which the local environment of the bacterium is modulated by its surrounding biofilm polymer. However, it is possible to speculate about these effects, on the basis of an understanding of polysaccharide chemistry. For example, such hygroscopic polymers could influence water activity, particularly in desiccating conditions (Mugnier and Jung, 1985; McEldowney and Fletcher, 1988). This in turn could influence various types of cellular activity, such as production of diacetyl by lactobacilli, accumulation of glycerol by *Dunaliella* spp. or of amino acids by various non-halophilic bacteria, and ethanol production by *Saccharomyces cerevisiae* (Mattiasson and Hahn-Hägerdal, 1982). Surfaces have also been found to protect bacteria from desiccation (Bushby and Marshall, 1977; McEldowney and Fletcher, 1988). This could be due to water retention by the bacterial polymers or by the surfaces themselves, such as occurs with clays (Bushby and Marshall, 1977).

An extremely important feature of the biofilm environment is that the microorganisms are immobilized in relatively close proximity to one another. Moreover, additional organisms, e.g., macroalgae, invertebrates, may be located within or on top of the biofilm matrix. Specific functional types of organisms may, through their activities, create conditions that favor other, complementary functional types. This would lead to the establishment of spatially separated, but interactive, functional groups of bacteria, which exchange metabolites at group boundaries. Such spatial segregation and functional complementarity is a feature of a number of environments. Examples are the stratification of anaerobes that occurs in aquatic sediments (Nedwell and Gray, 1987) or of phototrophs in algal mats (Ward et al., 1987).

Genetic exchange among organisms may also be favored in biofilms, as compared with aqueous phases. Plasmids encoding mercury resistance were transferred between bacteria in biofilms (Bale et al., 1987; Rochelle et al., 1989), and DNA transformation of *Bacillus subtilis* was found to be enhanced on the surfaces of sand grains (Lorenz et al., 1988).

3. Laboratory Investigations

There have been numerous laboratory studies addressing the influence of solid surfaces on the physiology of attached bacteria. Different types of metabolic activity have been measured, including assimilation of substrates, e.g., sugars (Fletcher, 1986), amino acids (Bright and Fletcher, 1983a,b; Dashman and Stotzky, 1986), and organic or fatty acids (Kjelleberg et al., 1982) and respiration of carbon substrates (Bright and Fletcher, 1983b; Stotzky, 1966a,b). Other ways of measuring activity have been determination of heat production by microcalorimetry (Gordon et al. 1983), and changes in intracellular components, e.g., internal organic phosphorus and sugar, measured by nuclear magnetic resonance (NMR)(Galazzo et al., 1987) or intracellular NADH, measured by fluorescence spectrophotometry (Doran and Bailey, 1987). Results from these diverse types of measurements have demonstrated that not only can the physiological activity of bacteria in biofilms differ from that of suspended cells, but also these differences in activity are expressed in a variety of ways. Moreover, it is not possible to generalize about the influence of surfaces on activity. In many cases, the effect appears to be favorable, e.g., increase in growth, substrate utilization, whereas in other cases, the opposite effect, or no effect, is observed.

4. Responses of Biofilm Bacteria to Ambient Conditions

Bacteria often respond to environmental factors or stimuli through changes in physiological processes or morphology. In some cases, the degree or nature of such responses has been found to be different for attached and freely suspended bacteria. For example, the growth and nitrite oxidation activity of *Nitrobacter* spp. on surfaces were monitored to evaluate the influence of pH changes on the bacteria. The specific growth rate of attached cells was higher than that of nonattached bacteria, and attached cells also exhibited less response to changes in pH value in continuous culture (Keen and Prosser, 1987).

Certain types of bacterial responses to stimuli appear to be expressed only on surfaces. For example, organized intercellular responses of particular types of bacteria have been observed on agar surfaces. With *Escherichia coli* growing on agar, during microcolony formation, the bacteria formed close side-by-side alignments, either through abrupt movements or asymmetric cell elongation (Shapiro and Hsu, 1989). Similarly, during cell division of *Pseudomonas fluorescens* on surfaces, the two daughter cells were observed to move apart laterally and then slide next to one another (Lawrence et al., 1987), in a manner similar to that observed with *E. coli* (Shapiro and Hsu, 1989).

Another type of bacterial behavior restricted to surfaces is the swarming motility of *Vibrio* and *Proteus* species. With *V. parahaemolyticus*, changes associated with swarming are the production of 100-1000 lateral flagella and cell elongation, resulting in cells 30-40 *u*m long. The lateral flagella enable the cells to translocate across the surface.

In an elegant study utilizing *lux* (denoting luminescence) gene fusions (Silverman et al., 1984; Belas et al., 1986), it was determined that the expression of the lateral flagella gene (*laf*) was stimulated not only by proximity of the solid surface, but also by increase in medium viscosity (Belas et al., 1986). Apparently, interference with motion of the single polar flagellum, which is characteristic of freely swimming cells, either by association with a solid surface or increase in medium viscosity, is recognized by the cell and translated into synthesis of lateral flagella.

5. Bacterial Survival in Biofilms

Bacteria in biofilms often appear to be protected from surrounding pertubations, and their survival may, in fact, be enhanced in biofilms, in comparison to the liquid phase. Such protection is particularly important in clinical situations, where biofilm bacteria are less sensitive to antibiotic treatment than free bacteria (Anwar et al., 1989; Gristina et al., 1989). Bacteria in biofilms have also been found to be more resistant to biocides, such as substituted phenols, biguanides, quaternary ammonium compounds (Gilbert and Brown, 1978, 1980) and chlorinated compounds (Herson et al., 1987; LeChevallier et al., 1988).

Two possible explanations have been provided for this enhanced resistance exhibited by biofilm bacteria. First, the bacteria may be protected, to some extent, by the intercellular polymeric matrix, which may retard diffusion of antibiotics or biocides to any depth (Costerton et al., 1985). Second, bacteria on surfaces are physiologically different from free bacteria, and they may be relatively inactive when embedded in biofilms (Brown et al., 1988). The microenvironment within the biofilm is substantially different from that in the aqueous phase, and one result of this might be the observed decrease in bacterial activity (Brown et al., 1988), as well as resistance. Specific bacterial components that could be modified by ambient conditions, such as those existing within a biofilm, include outer-membrane proteins (Griffiths et al., 1983), such as porins (Lugtenberg and van Alphen, 1983), lipopolysaccharides (Dean et al., 1977), outer-membrane phospholipids (Minnikin et al., 1974; Cozens and Brown, 1983), and cation content (Kenward et al., 1979). Such modifications could affect antibiotic sensitivity. Moreover, some antibiotics (e.g., *B*-lactams) are active only with growing cells, and thus would not be effective with slow- or non-growing bacteria in biofilms (Tuomanen et al., 1986).

6. Biofilms in Natural Environments

6.1. BACTERIA ASSOCIATED WITH PARTICLES

There is considerable interest in the activity of bacteria associated with suspended particles in aquatic environments, particularly coastal regions, because of their potential

role in the breakdown of particulate detritus. Such particles include resuspended sediment (Ducklow et al., 1982), dead and senescent phytoplankton and zooplankton, and coagulated organic material (Kepkay and Johnson, 1988). The activity of particle-associated bacteria has been measured in a variety of ways, including determination of respiration (Harvey and Young, 1980); assimilation of radiolabelled substrates (Hodson et al., 1981; Kirchman and Mitchell, 1982; Hollibaugh and Azam, 1983), including tritiated thymidine (Kirchman, 1983; Edwards and Meyer, 1986), frequency of dividing cells (Pedros-Alio and Brock, 1983), and extracellular enzyme activity (Hoppe et al., 1983; Vives-Rego et al., 1985).

Frequently, activity has been found to be largely associated with the particle fraction (Harvey and Young, 1980; Kirchman and Mitchell, 1982; Hollibaugh and Azam, 1983; Vives-Rego et al., 1985; Edwards and Meyer, 1986). Activity of attached bacteria has often exceeded that of suspended bacteria on a per-cell basis (Hodson et al., 1981; Kirchman and Mitchell, 1982; Paerl and Merkel, 1982; Iriberri et al., 1987; Simon, 1988). However, on a per-volume basis, activity of particle-associated and free cells may often be similar, because of the tendency for attached cells to have larger volumes (Hodson et al., 1981).

Other investigations determined that activities of particle-associated and free cells were similar (Iriberri et al., 1990), or dependent upon the method used to measure activity. For example, with heterotrophic bacteria from Lake Mendota, Wisconsin, attached bacteria assimilated more acetate than free cells, whereas free bacteria assimilated more sulfate (Pedros-Alio and Brock, 1983).

6.2. BIOFILMS ON SUBMERGED SURFACES

Bacteria that occur on submerged solid surfaces play an important role in community dynamics, biofouling, and microbially induced corrosion (Hamilton, 1985). These are complex communities, and are likely to foster numerous synergistic, mutualistic, competitive, and antagonistic interactions among their members. Microalgae and cyanobacteria will occur where light is available, and in oligotrophic environments, heterotrophic bacterial activity may be considerably dependent upon photosynthetic activity in the biofilm (Geesey et al., 1978; Haack and McFeters, 1982; Lock and Ford, 1985). In more nutrient-rich systems, biofilm build-up is largely due to heterotrophic activities.

The organic components in water may have a complex influence on biofilm physiology. Microcalorimetry was used to evaluate the activity of river bacteria, and it was found that bacterial activity was stimulated by removal of the organic fraction with a molecular weight greater than 1000 (Lock and Ford, 1986). This fraction appeared to inhibit activity of both heterotrophic biofilm bacteria and biofilm communities with both heterotrophs and autotrophs.

Because biofilm communities tend to be complex, both taxomically and functionally, there is considerable potential for synergistic interactions among the

constituent organisms. Thus, there is the fostering of the development of homeostatic mechanisms that could protect the bacteria from outside perturbations. Such balancing mechanisms would be extremely important in natural communities that are exposed to disturbances such as pollution from industrial, agricultural, and domestic sources. A kinetic analysis of degradation of sodium dodecyl sulfate by biofilm and planktonic communities provided evidence of such stabilization in biofilms (Anderson et al., 1990). The biofilm activity fluctuated less that that of planktonic populations when exposed to different temperatures or water compositons.

7. Conclusions

The physiological activities of biofilm bacteria frequently differ from those of freely suspended cells. In part, this is due to the influence of hydrodynamic factors, e.g., flow, mass transfer, and physicochemical characteristics of the solid-liquid interface, e.g., adsorption of substrates. However, the reasons for altered physiological activity in biofilms are clearly complex and involve the architectural and chemical complexity of the biofilm structure. The intercellular polymeric matrix allows the development of a multicellular structure that is not unlike a tissue. This fosters the development of synergistic interactions among organisms and homeostatic mechanisms that help to preserve the overall community. It is also possible that, in some cases, intracellular homeostatic mechanisms are more effective for cells on surfaces. Evidence for such stability has been provided by both laboratory (Doran and Bailey, 1987; Keen and Prosser, 1988) and *in situ* (Anderson et al., 1990) studies. Furthering our knowledge of the underlying mechanisms of biofilm metabolism and homeostasis will enhance our abilities to facilitate preservation of natural communities that are stressed with pollution and to deal effectively with hazardous biofilms, such as those in clinical settings.

8. References

Anderson, D.J., Day, M.J., Russell, N.J. and White, G.F. (1990) 'Die-away kinetic analysis of the capacity of epilithic and planktonic bacteria from clean and polluted river water to biodegrade sodium dodecyl sulfate', Appl. Environ. Microbiol. 56, 758-763.

Anwar, H., van Biesen, T., Dasgupta, M., Lam, K. and Costerton, J.W. (1989) 'Interaction of biofilm bacteria with antibiotics in a novel *in vitro* chemostat system', Antimicrob. Agents Chemother. 33, 1824-1826.

Bale, M.J., Fry, J.C. and Day, M.J. (1987) 'Plasmid transfer between strains of *Pseudomonas aeruginosa* on membrane filters attached to river stones', J. Gen. Microbiol. 133, 3099-3107.

Bayer, E.A., Setter, E. and Lamed, R. (1985) 'Organization and distribution of the cellulosome in *Clostridium thermocellum*', J. Bacteriol. 163, 552-559.

Belas, R., Simon, M. and Silverman, M. (1986) 'Regulation of lateral flagella gene transcription in *Vibrio parahaemolyticus*', J. Bacteriol. 167, 210-218.

Bright, J.J. and Fletcher, M. (1983a) 'Amino acid assimilation and electron transport system activity in attached and free-living marine bacteria', Appl. Environ. Microbiol. 45, 818-825.

Bright, J.J. and Fletcher, M. (1983b) 'Amino acid assimilation and respiration by attached and free-living populations of a marine *Pseudomonas* sp.', Microb. Ecol. 9, 215-226.

Brown, M.R.W., Allison, D.G. and Gilbert, P. (1988) 'Resistance of bacterial biofilms to antibiotics: a growth related effect?', J. Antimicrob. Chemother. 22, 777-783.

Bushby, H.V.A. and Marshall, K.C. (1977) 'Water status of rhizobia in relation to their susceptibility to desiccation and to their protection by montmorillonite', J. Gen. Microbiol. 99, 19-27.

Characklis, W.G. (1984) 'Biofilm development: a process analysis', in K.C. Marshall (ed.), Microbial Adhesion and Aggregation, Springer-Verlag, Berlin, pp. 137-157.

Characklis, W.G. and Marshall, K.C. (1990) Biofilms, John Wiley, New York.

Costerton, J.W., Marrie, T.J. and Cheng, K.-J. (1985) 'Phenomena of bacterial adhesion', in D.C. Savage and M. Fletcher (eds.), Bacterial Adhesion, Plenum Press, New York, pp. 3-43.

Cozens, R.M. and Brown, M.R.W. (1983) 'Effect of nutrient depletion on the sensitivity of *Pseudomonas cepacia* to antimicrobial agents', J. Pharmaceu. Sci. 72, 1363-1365.

Dashman, T. and Stotzky, G. (1986) 'Microbial utilization of amino acids and a peptide bound on homoionic montmorillonite and kaolinite', Soil Biol. Biochem. 18, 5-14.

Dean, A.C.R., Ellwood, D.C., Melling, J. and Robinson, A. (1977) 'The action of antibacterial agents on bacteria grown in continuous culture', in A.C.R. Dean, D.C. Ellwood, C.G.T. Evan and J. Melling (eds), Continuous Culture, vol. 6, Ellis Horwood, Chichester, UK, pp. 251-261.

Doran, P.M. and Bailey, J.E. (1987) 'Effects of immobilization on growth, fermentation properties, and macromolecular composition of *Saccharomyces cerevisiae* attached to gelatin', Biotechnol. Bioengin. 28, 73-87.

Ducklow, H.W., Kirchman, D.L. and Rowe, G.T. (1982) 'Production and vertical flux of attached bacteria in the Hudson River Plume of the New York Bight as studied with floating sediment traps', Appl. Environ. Microbiol. 43, 769-776.

Edwards, R.T. and Meyer, J.L. (1986) 'Production and turnover of planktonic bacteria in two southeastern blackwater rivers', Appl. Environ. Microbiol. 52, 1317-1323.

Estermann, E.F., Peterson, G.H. and McLaren, A.D. (1959) 'Digestion of clay-protein, lignin-protein and silica-protein complexes by enzymes and bacteria', Soil Sci. Soc. Amer. Proc. 23, 31-36.

Fletcher, M. (1986) 'Measurement of glucose utilization by *Pseudomonas fluorescens* that are free-living and that are attached to surfaces', Appl. Environ. Microbiol. 52, 672-676.

Galazzo, J.L., Shanks, J.V. and Bailey, J.E. (1987) 'Comparison of suspended and immobilized yeast metabolism using ^{31}P nuclear magnetic resonance spectroscopy', Biotechnol. Technol. 1, 1-6.

Geesey, G.G., Mutch, R., Costerton, J.W. and Green, R.B. (1978) 'Sessile bacteria: an important component of the microbial population in small mountain streams', Limnol. Oceanogr. 23, 1214-1223.

Gilbert, P. and Brown, M.R.W. (1978) 'Influence of growth rate and nutrient limitation on the gross cellular composition of *Pseudomonas aeruginosa* and its resistance to 3- and 4-chlorophenol', J. Bacteriol. 133, 1066-1072.

Gilbert, P. and Brown, M.R.W. (1980) 'Cell-wall mediated changes in the sensitivity of *Bacillus megaterium* to chlorhexidine and 2-phenoxyethanol, associated with growth rate and nutrient limitation', J. Appl. Bacteriol. 48, 223-230.

Gordon, A.S., Gerchakov, S.M. and Millero, F.J. (1983) 'Effects of inorganic particles on metabolism by a periphytic marine bacterium', Appl. Environ. Microbiol. 45, 411-417.

Griffiths, E., Stevenson, P. and Joyce, P. (1983) 'Pathogenic *Escherichia coli* express new outer membrane proteins when growing *in vivo*', FEMS Microbiol. Lett. 16, 95-99.

Gristina, A.G., Jennings, R.A., Naylor, P.T., Myrvik, Q.N. and Webb, L.X. (1989) 'Comparative *in vitro* antibiotic resistance of surface-colonizing coagulase-negative staphylococci', Antimicrob. Agents Chemother. 33, 813-816.

Haack, T.K. and McFeters, G.A. (1982) 'Nutritional relationships among microorganisms in an epilithic biofilm community', Microb. Ecol. 8, 115-126.

Hamilton, W.A. (1985) 'Sulphate-reducing bacteria and anaerobic corrosion', Ann. Rev. Microbiol. 39, 195-217.

Harvey, R.W. and Young, L.Y. (1980) 'Enumeration of particle-bound and unattached respiring bacteria in the salt marsh environment', Appl. Environ. Microbiol. 40, 156-160.

Herson, D.S., McGonigle, B., Payer, M.A. and Baker, K.H. (1987) 'Attachment as a factor in the protection of *Enterobacter cloacae* from chlorination', Appl. Environ. Microbiol. 53, 1178-1180.

Hodson, R.E., Maccubbin, A.E. and Pomeroy, L.R. (1981) 'Dissolved adenosine triphosphate utilization by free-living and attached bacterioplankton', Mar. Biol. 64, 43-51.

Hoffman, A.S. (1986) 'Letter to the Editor: a general classification scheme for "hydrophilic" and "hydrophobic" biomaterial surfaces', J. Biomed. Mat. Res. 20, ix-xi.

Hollibaugh, J.T. and Azam, F. (1983) 'Microbial degradation of dissolved proteins in seawater', Limnol. Oceanogr. 28, 1104-1116.

Holt, D.M. and Jones, E.B.G. 1983) 'Bacterial degradation of lignified wood cell walls in anaerobic aquatic habitats', Appl. Environ. Microbiol. 46, 722-727.

Hoppe, H.-G., Gocke, K., Zamorano, D. and Zimmermann, R. (1983) 'Degradation of macromolecular organic compounds in a tropical lagoon (Cienaga Grande,

Colombia) and its ecological significance', Internat. Rev. gesamt. Hydrobiol. 68, 811-824.

Iriberri, J., Unanue, M., Barcina, I. and Egea, L. (1987) 'Seasonal variation in population density and heterotrophic activity of attached and free-living bacteria in coastal waters', Appl. Environ. Microbiol. 53, 2308-2314.

Iriberri, J. Unanue, M., Ayo, B., Barcina, I. and Egea, L. (1990) 'Bacterial production and growth rate estimation from [^{3}H]thymidine incorporation for attached and free-living bacteria in aquatic systems', Appl. Environ. Microbiol. 56, 483-487.

Keen, G.A. and Prosser, J.I. (1987) 'Interrelationship between pH and surface growth of *Nitrobacter*', Soil Biol. Biochem. 19, 665-672.

Keen, G.A. and Prosser, J.I. (1988) 'The surface growth and activity of *Nitrobacter*', Microb. Ecol. 15, 21-39.

Kenward, M.A., Brown, M.R.W. and Fryer, J.J. (1979) 'The influence of calcium or magnesium on the resistance to EDTA, polymyxin B or cold shock, and the composition of *Pseudomonas aeruginosa* in glucose or magnesium depleted batch cultures', J. Appl Bacteriol. 47, 489-503.

Kepkay, P.E. and Johnson, B.D. (1988) 'Microbial response to organic particle generation by surface coagulation in seawater', Mar. Ecol. Progr. Ser. 48, 193-198.

Kirchman, D. (1983) 'The production of bacteria attached to particles suspended in a freshwater pond', Limnol. Oceanogr. 28, 858-872.

Kirchman, D. and Mitchell, R. (1982) 'Contribution of particle-bound bacteria to total microheterotrophic activity in five ponds and two marshes', Appl. Environ. Microbiol. 43, 200-209.

Kjelleberg, S., Humphrey, B.A. and Marshall, K.C. (1982) 'Effect of interfaces on small, starved marine bacteria', Appl. Environ. Microbiol. 43, 1166-1172.

Lamed, R., Naimark, J., Morgenstern, E. and Bayer, E.A. (1987) 'Specialized cell surface structures in cellulolytic bacteria', J. Bacteriol. 169, 3792-3800.

Lawrence, J.R., Delaquis, P.J., Korber, D.R. and Caldwell, D.E. (1987) 'Behavior of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments', Microb. Ecol. 14, 1-14.

LeChevallier, M.W., Cawthon, C.D. and Lee, R.G. (1988) 'Inactivation of biofilm bacteria', Appl. Environ. Microbiol. 54, 2492-2499.

Lock, M.A. and Ford, T.E. (1985) 'Microcalorimetric approach to determine relationships between energy supply and metabolism in river epilithon', Appl. Environ. Microbiol. 49, 408-412.

Lock, M.A. and Ford, T.E. (1986) 'Metabolism of dissolved organic matter by attached microorganisms in rivers', in F. Megusar and M. Gantar (eds.), Perspectives in Microbial Ecology, Slovene Society for Microbiology, Ljubljana, Yugoslavia, pp.367-374..

Lorenz, M.G. and Wackernagel, W. (1987) 'Adsorption of DNA to sand and variable degradation rates of adsorbed DNA', Appl. Environ. Microbiol. 53, 2948-2952.

Lorenz, M.G., Aardema, B.W., and Krumbein, W.E. (1981) 'Interaction of marine sediment with DNA and DNA availability to nucleases', Mar. Biol. 64, 225-230.

Lorenz, M.G. Aardema, B.W., and Wackernagel, W. (1988) 'Highly efficient genetic transformation of *Bacillus subtilis* attached to sand grains', J. Gen. Microbiol. 134, 107-112.

Lugtenberg, B. and van Alpen, L. (1983) 'Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria', Biochim. Biophys. Acta 737, 51-115.

McEldowney, S. and Fletcher, M. (1988) 'The effect of temperature and relative humidity on the survival of bacteria attached to dry solid surfaces', Lett. Appl. Microbiol. 7, 83-86.

Mattiasson, B. and Hahn-Hägerdal, B. (1982) 'Microenvironmental effects on metabolic behaviour of immobilized cells: a hypothesis', Eur. J. Appl. Microbiol. Biotechnol. 16, 52-55.

Minnikin, D.E., Abdulrahimzadeh, H. and Baddiley, J. (1974) 'Replacement of acidic phospholipids by acidic glycolipids in *Pseudomonas diminuta*', Nature, Lond. 249, 268-269.

Mugnier, J. and Jung, G. (1985) 'Survival of bacteria and fungi in relation to water activity and the solvent properties of water in biopolymer gels', Appl. Environ. Microbiol. 50, 108-114.

Munch, J.C. and Ottow, J.C.G. (1982) 'Einfluss von Zellkontakt und Eisen(III)-Oxidform auf die Bacterielle Eisenreduktion', Zeit. Pflanzen. Dung. Bodenkunde 145, 66-77.

Nedwell, D.B. and Gray, T.R.G. (1987) 'Soils and sediments as matrices for microbial growth', in M. Fletcher, T.R.G.Gray, and J.G. Jones (eds.), Ecology of Microbial Communities, Cambridge University Press, Cambridge, UK, pp. 21-54..

Norde, W. (1986) 'Adsorption of proteins from solution at the solid-liquid interface', Adv. Coll. Interface Sci. 25, 267-340.

Ogram, A.V., Jessup, R.E., Ou, L.T. and Rao, P.S.C. (1985) 'Effects of sorption on biological degradation rates of (2,4-dichlorophenoxy)acetic acid in soils', Appl. Environ. Microbiol. 49, 582-587.

Paerl, H.W. and Merkel, S.M. (1982) 'Differential phosphorus assimilation in attached vs. unattached microorganisms', Arch. Hydrobiol. 93, 125-134.

Pedros-Alio, C. and Brock, T.D. (1983) 'The importance of attachment to particles for planktonic bacteria', Arch. Hydrobiol. 98, 354-379.

Quiquampoix, H. (1987) 'A stepwise approach to the understanding of extracellular enzyme activity in soil. I. Effect of electrostatic interactions on the conformation of a *B*-D-glucosidase adsorbed on different mineral surfaces', Biochim. 69, 753-763.

Quiquampoix, H., Chassin, P. and Ratcliffe, R.G. (1989) 'Enzyme activity and cation exchange as tools for the study of the conformation of proteins adsorbed on mineral surfaces', Prog. Colloid Polym. Sci. 79, 1-5.

Rochelle, P.A., Fry, J.C. and Day, M.J. (1989) 'Factors affecting conjugal transfer of plasmids encoding mercury resistance from pure cultures and mixed natural suspensions of epilithic bacteria', J. Gen. Microbiol. 135, 409-424.

Shapiro, J.A. and Hsu, C. (1989) '*Escherichia coli* K-12 cell-cell interactions seen by time-lapse video', J. Bacteriol. 171, 5963-5974.

Silverman, M., Belas, R. and Simon, M. (1984) 'Genetic control of bacterial adhesion', in K.C. Marshall (ed.), Microbial Adhesion and Aggregation, Springer-Verlag, Berlin, pp. 95-107.

Simon, M. (1988) 'Growth characteristics of small and large free-living and attached bacteria in Lake Constance', Microb. Ecol. 15, 151-163.

Stotzky, G. (1966a) 'Influence of clay minerals on microorganisms. II. Effect of various clay species, homoionic clays, and other particles on bacteria', Can. J. Microbiol. 12, 831-848.

Stotzky, G. (1966b) 'Influence of clay minerals on microorganisms. III. Effect of particle size, cation exchange capacity, and surface area on bacteria', Can. J. Microbiol. 12, 1235-1246.

Takakuwa, S., Fujimori, T. and Iwasaki, H. (1979) 'Some properties of cell-sulfur adhesion in *Thiobacillus thiooxidans*', J. Gen. Appl. Microbiol. 25,21-29.

Tuomanen, E., Cozens, R., Tosch, W., Zak, O. and Tomasz, A. (1986) 'The rate of killing of *Escherichia coli* by *B*-lactam antibiotics is strictly proportional to the rate of bacterial growth', J. Gen. Microbiol. 132, 1297-1304.

Vives-Rego, J., Billen, G., Fontigny, A. and Somville, M. (1985) 'Free and attached proteolytic activity in water environments', Mar. Ecol. Prog. Ser. 21, 245-249.

Ward, D.M., Tayne, T.A., Anderson, K.L. and Bateston, M.M. (1987) 'Community structure and interactions among community members in hot spring cyanobacterial mats', in M. Fletcher, T.R.G. Gray, and J.G. Jones (eds.), Ecology of Microbial Communities, Cambridge University Press, Cambridge, UK, pp. 179-210.

Weber, J.B. and Coble, H.D. (1968) 'Microbial decomposition of diquat adsorbed on montmorillonite and kaolinite clays', J. Agron. Food Chem. 16, 475-478.

Zvyagintseva, I.S. and Zvyagintsev, D.G. (1969) 'Effect of microbial cell adsorption onto steroid crystals on the transformation of the steroid', Microbiology 38, 691-694.

INFLUENCE OF SURFACES ON MICROBIAL ACTIVITY

N. MOZES and P.G. ROUXHET
Université Catholique de Louvain
Unité de Chimie des Interfaces
Place Croix du Sud 2/18
1348 Louvain-La-Neuve
Belgium

1. Introduction

Three zones can be roughly distinguished in biofilms: (*i*) the primary layer of cells which are in contact with the solid substratum; (*ii*) the bulk of the biofilm; (*iii*) the surface layer of cells which are in direct contact with the liquid phase. The activity of the cells in each one of these zones will be affected to different degrees by mass transfer limitations, by physico-chemical interactions with the support, or by shear forces exerted by the liquid medium.

Surfaces may affect attached cells through a number of mechanisms. Solid surfaces may trigger an internal physiological response that will affect cell morphology (Silverman *et al.*, 1984) and behaviour (Burchard, 1981). Surfaces and mass transfer limitations due to confinement create an environment where local concentrations of ions, small molecules or polymers are different from the concentrations in the bulk of the solution; the chemical species concerned may be substrates for the cells or toxic agents.

Contradictory reports can be found in the literature regarding the physiological activity of bacteria attached to solid surfaces as compared to bacteria freely suspended in the bulk aqueous phase (Durand and Navarro 1978; Ellwood *et al.*, 1982; Klein and Ziehr, 1990; Van Loosdrecht *et al.*, 1990). This is partly due to the variability in the systems under investigation: the type of organisms and the measured parameter. The criterion used by different authors for "activity" is sometimes growth rate, half-life time, oxygen consumption, glucose consumption, CO_2 release, ethanol (or other metabolite) production, a specific enzymatic activity, *etc*. Results are also expressed in various ways.

Morisaki (1983) showed that while the respiratory activity (O_2 uptake rate) of *E. coli* was enhanced in presence of hydrophobic solid particles, the uptake rate of the substrate (glucose) was depressed. He noted that, in his experimental conditions (absence of nitrogen), no cell growth could occur; therefore all the glucose consumed was used via respiration and production of ATP for maintenance. The situation could be different under conditions allowing cellular growth where glucose would be oxidized only partly and intermediate metabolites would be used as building material. Mattiasson and Hahn-Hagerdal (1982) also suggested that the micro-environment of immobilized cells may favour maintenance metabolism at the expenses of cell growth.

The aim of this chapter is to recall and discuss a few examples reported in the literature, rather than to give an extensive up to date review of the subject. The reader is referred to the recent reviews of Klein and Ziehr (1990) and Van Loosdrecht *et al.* (1990).

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 125–136.

2. Solid Surfaces Triggering Physiological Response

A given species has genetic information for a large repertoire of properties; the encounter between a population of these bacteria and a particular surface, may influence the expression of the genetic information. Thus the production of microfibrils by *Rhizobium* and *Agrobacterium* appears to be a response to a signal resulting from the interactions between the bacteria and a plant surface (Dazzo, 1984).

The essence of responsive control is information processing, whereby the bacterium senses some signal in the environment and responds accordingly. The bacterium can indirectly perceive where it is by sensing temperature, osmotic conditions, or metabolites, but one may wonder whether a micro-organism can sense a surface as such? With higher organisms the direct interaction of the cell membrane with a surface influences division and differentiation, but it is not clear that cell membrane processes in microbial cells can be modified by elastic deformation of the cell envelope, or that micro-organisms such as bacteria can sense and respond to direct contact between their cell envelope and a surface. Karel *et al.* (1990) mentioned that contact with foreign surfaces may be sensed by membrane receptors that respond to the physical state of the cell envelope, such as local loss of tension in the cell wall; a prototype for such receptor would be the *Env*Z protein that modulates osmotic responses. One has to distinguish between "variable control", meaning that an effect is produced by a subset of a heterogeneous population that takes over in certain conditions, and "response control", meaning physiological change triggered by an external signal.

Dagostino *et al.* (1991) attempted to demonstrate the range of physiological responses in bacteria attached to surfaces and tried to determine whether the "switching-on" of some genes is a direct response to the physical presence of a solid. They employed transposon mutagenesis to insert a marker gene from *Escherichia coli* into *Deleya marina* or *Pseudomonas* recipients and managed to select transconjugates in which the marker gene (*lac*Z) was expressed by the bacteria on a polystyrene surface, but not in liquid or on agar surface. The mechanism of this "switching-on" effect has not yet been elucidated.

2.1. APPENDAGES

Silverman *et al.* (1984) described the behaviour of *Vibrio parahaemolyticus* . This bacterium produces a single, polar sheathed flagellum in liquid media, but numerous, lateral non-sheathed flagella on agar. It is clear that a new appendage is produced after growth in contact with a surface, but the question whether the bacterium actually possesses a tactile sense remains to be solved. A genetic analysis of the lateral flagella system has been carried out in order to explore the sensory aspect of surface perception which triggers the structural change. Transposon mutagenesis and recombinant DNA methods provided sensitive, direct, and convenient means to measure expression of lateral flagella genes. It was found that the surface-dependent turn-on of lateral flagella gene expression did not occur immediately, as might be expected if these bacteria were to have tactile sense. Instead, 60 to 90 min elapsed before transcription began. This was interpreted as meaning that the cells must have time to synthesize and accumulate some compound which acts as signal for initiation of transcription of the lateral flagella gene (see also Fletcher,1992).

Hall and Creig (1983) described a similar situation for *Azospirillum brasilense* which possesses a single polar flagellum and also numerous thinner lateral flagella. Studies of mutants lacking either polar or lateral flagella, showed that the former is responsible for swimming motility

in liquid media, and the latter are responsible for swarming on semi-solid media. A solid surface is required for production of the lateral flagella. Swarming was dependent on the degree of hardness of the supporting gel: it occurred optimally at agar concentrations ranging from 0.40% to 0.75%; an incubation at 30°C, on a 0.75% gel, led to a greater degree of swarming than at 37°C, on the same agar. The interpretation was that the signal felt by the bacterium is the increased viscosity that restricts the movement of the polar flagellum. Chemical agents such as ethylenediamine tetraacetic acid, p-nitrophenyl glycerol, sodium deoxycholate and sodium taurocholate did not inhibit lateral flagella formation, but could inhibit swarming, while NaCl and Na_2SO_4 inhibited both formation of lateral flagella and swarming.

2.2. DWARFTING

Starvation of certain marine bacteria leads to the formation of small cells with a greater survival and adhesive capacities. Kjelleberg *et al.* (1982, 1983) and Humphrey and Marshall (1984) studied the starvation induced response of *Vibrio* DW1 and the activity of the bacteria in the liquid phase and at the solid/liquid interface. The presence of a solid surface has been shown to induce several responses in the absence of an energy substrate. Cell size reduction, which occurred in the initial period of starvation, was more marked at the solid/liquid interface than in the liquid phase. The metabolic activity taking place during this dwarfting phase, and detected by oxygen uptake and heat production, was higher for the surface-associated cells than for the cells at the liquid phase. The small starved bacteria did not grow in the liquid phase nor at the liquid/solid interface; addition of a nutrient rich broth led to a quick return to normal size, motility and division only if a surface was present. The authors suggested that the dwarfting response of these cells at interfaces may be important for survival under conditions of nutritional limitation. It was postulated that the surface triggered a burst of activities during which starving cells reorganized their morphology to an optimal surviving configuration (highest surface /volume ratio) and consumed endogenous substrates in the process.

2.3. GLIDING

Gliding motility is found in several diverse genera of bacteria. The gliding cell remains in contact with a surface but undergoes translational motion. According to Burchard (1981), no wringgling, contraction or peristaltic alterations are visible during the translocation along solid bodies. Gliding movements are not always regular, but intermittent and hesitant, with frequent changes of direction; they may be random or oriented. No obvious locomotory organelles could be related to the phenomenon, but association with a surface is considered a requisite. Most gliders have in common a Gram-negative type cell envelope. They can move as individual cells or in multicellular configuration. The velocity of gliding movement varies widely among the species (1-2 μm/min for *Myxococcus*; 600 μm/min for *Oscillatoria*). The velocity of gliders is affected by moisture level, temperature, nature of the substratum, and association with other bacteria. Among the various mechanisms which have been proposed to explain the gliding motility are osmotic forces, surface tension phenomena, slime secretion, contractile waves. The movement may be oriented by physical, chemical and biological factors which may be beneficial to the glider. Gliding bacteria etch agar surfaces, leaving trails. When a glider encounters at a low angle a groove previously etched in agar by another glider, it tends to follow the groove and glide in it at greater velocity.

Humphrey *et al.* (1979) attributed gliding of *Flexibacter* to the secretion of a viscous glycoproteinic slime by the bacteria. The slime facilitates translational motion; however, it

prevents separation of the cell from the substratum, because the work against viscosity required for such a separation would be too high.

It was suggested that the nature of the substratum is a key factor in the adhesion and motility of gliding bacteria. Since a variety of such bacteria adhere tenaciously to hydrophobic substrata and weakly to hydrophilic surfaces, Sorongon *et al.* (1991) proposed that their adhesion involves, at least in part, hydrophobic interactions. They studied the hydrophobicity of the surfaces of several gliding bacteria and several adhesion-defective mutants and revertants. Attempts were made to correlate the results with adhesion to a moderately hydrophobic substratum and with changes in arrays of proteins exposed at the cell surface, but no conclusive correlations could be demonstrated.

3. The Micro-environment at Close Proximity of Surfaces

3.1. INTERFACE AS A POOL OF NUTRIENTS

Adsorption of molecules or ions leads to higher concentrations at the solid/liquid interface as compared to the liquid phase. The solid/liquid interface in a natural environment was described by a few authors as a pool of nutrients. Adhesion of starved bacteria and scavenging of adsorbed nutrients appears thus as a survival mechanism in natural environment (Kjelleberg *et al.*, 1983; Kefford *et al.*, 1986). Organic compounds adsorbed at surfaces are considered to serve as a concentrated source of nutrients. This may be crucial in nature where the liquid phase is nutritionally poor. In man-made situations (bioreactors with immobilized cells, waste water treatment plants, laboratory experiments) the liquid medium is generally rather rich in nutrient and adsorption of the substrate by a surface does not improve its availability for the attached cells (see also Fletcher, 1992).

The adsorbed phase may thus act as a reservoir of nutrients. However, if it is in thermodynamic equilibrium with the solution phase, then the chemical potential of ions and molecules is the same in both phases. Therefore, the driving force determining their tendency to be involved in a chemical reaction or in a physico-chemical process is also the same.

Direct transfer may occur between the adsorbed layer and the fraction of the cell surface which is in close contact with the substratum surface. Nevertheless, one must keep in mind real dimensions (Rouxhet and Mozes, 1990). Due to the rounded shape of the micro-organism, only a very small fraction of the cell surface is in direct contact with the support surface. The thickness of the layer of small adsorbed molecules may be of the order of 1 nm while the cell diameter is of the order of 1 μm. Therefore, unless the cell spreads on the surface, which occurs for animal but not for microbial cells, most of the cell surface is exposed to the bulk solution.

The amount of substance adsorbed on the surface covered by a microbial cell is very small compared to the metabolic needs of the cell (Rouxhet and Mozes, 1990). Consider a 1 nm thick layer (the approximate length of a disaccharide) of a compound with a molecular weight of 100 $g.mol^{-1}$ and a specific weight of 1 $g.cm^{-3}$. If the attached cell covers a surface of 1 μm^2 and is characterized by a specific uptake rate of 10^{-18} $mol.cell^{-1}.s^{-1}$, which is in the range reported for oxygen consumption by bacteria, the amount of substrate adsorbed on the area of the surface occupied by the cell will be consumed within 10 seconds. The more or less direct transfer between a non-porous support and an attached cell is thus expected to be of marginal importance, unless the uptake is very slow, or the substrate is a macromolecule forming a thick adsorbed layer.

A similar point was made by Ellwood *et al.* (1982) who summarized literature observations suggesting that surfaces have, intrinsically, a positive influence on the growth of microorganisms. They added their own results, obtained in chemostat enrichment studies: faster growth and a more variable population were observed at the surface. This indicated that the solid surface provided an environment niche different from that in free fluid culture. The authors rejected the generally accepted explanation of surface-enhanced growth by an increased concentration of a limiting nutrient at the surface. Their argument was that the increased concentration of the limiting substrate would be soon consumed, unless its mass transfer from the bulk phase to the solid/liquid interface was continuous and rapid. They proposed an alternative interpretation based on the Mitchell chemiosmotic theory. Protons expelled by a cell into the aqueous phase are free to diffuse away, whereas for a cell near the substratum surface protons accumulate at the side of the cell facing the substratum. These accumulating protons alter the proton motive force across the plasma membrane of the bacterium. The authors speculated that it is energetically advantageous for organisms to be attached to, and to interact with, a surface, hence the increased metabolic activity associated with growth at surfaces. This kind of explanation is typically an indirect effect of the surface.

3.2. INFLUENCE OF SURFACE ELECTRIC POTENTIAL

Consider a negatively charged surface with an electric potential ψ_d. According to the diffuse double layer theory the potential exerted in the solution decreases as a function of the distance,x, from the surface following :

$$\psi = \psi_d \exp(-\kappa x) \qquad (1)$$

where κ^{-1} is often called the "thickness" of the diffuse double layer. The existence of potential ψ at a given spot modifies the distribution of ion activities (M)

$$(M)_S = (M)_1 \exp(-z F\psi/RT) \qquad (2)$$

where subscripts 1 and s designate the liquid phase and the spot considered near the surface, respectively; z, F, R and T are the charge of ion M, the Faraday constant, the gas constant and the absolute temperature, respectively. It turns out that the pH in the vicinity of a negative surface is lower than in the free solution.

An increase of pH optimum was observed (Hattori and Furusaka, 1960; Hattori and Hattori, 1987) for the oxidation of various substrates by bacteria attached to Dowex 1 particles, compared to free cells (Figure 1, left). As the support surface was positively charged, one proposed explanation was that accumulation of negatively charged cells produced a negative electric potential in their environment, the local pH being smaller than the pH measured in the free solution.

Electrostatic interactions and local pH effects should be considered with caution, taking the two following remarks into consideration.

1- Although the pH may be different near a surface, as compared with the free solution, the molar free enthalpy G, *i.e.* the tendency of proton to react, is the same in the surface phase and in the solution when they are at equilibrium :

$$G_S = \mu^\circ + RT \ln (H^+)_S + F\psi/RT = G_1 = \mu^\circ + RT \ln (H^+)_1 \qquad (3)$$

2- Although the proton reactivity is not modified, the "local pH" term may be taken as a loose expression to mention the influence of a charged surface. However the "thickness" of the diffuse double layer κ^{-1}, which characterizes the rate of decrease of the potential *vs.* distance, is about 10 nm at an ionic strength of 10^{-3} and varies inversely with the square root of the ionic strength. Figure 2 shows that the potential exerted by a flat surface may only affect a small fraction of the cell volume or surface.

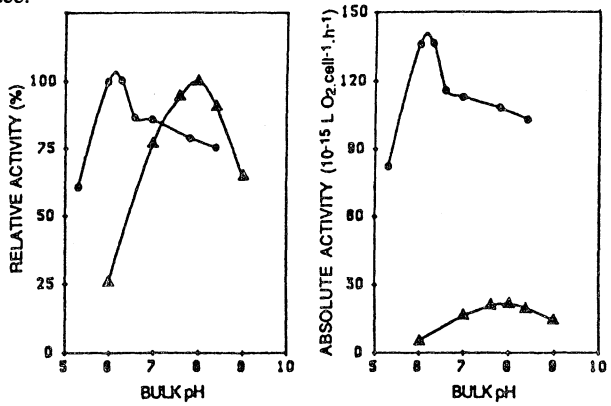


Figure 1. Oxidation of succinate by free (●) and immobilized (▲) *Escherichia coli* : left, relative activity obtained by normalization of each set of data; right, absolute activity in 10^{-15} $LO_2.cell^{-1}.h^{-1}$ (Hattori and Hattori, 1987).

In fact a shift of pH optimum as a result of immobilization of micro-organisms (or enzymes) must be considered critically when it is based on different sets of data, which may differ appreciably but are normalized by taking the highest value of each set as 100 %. Figure 1 shows indeed that the main line of interpretation may be very different depending on whether relative or absolute activities are taken into consideration. When the latter are used, the striking point is the lower activity of immobilized cells, compared to free cells. This may be attributed to diffusion limitations, and further discussion of the influence of surface potential is then not justified.

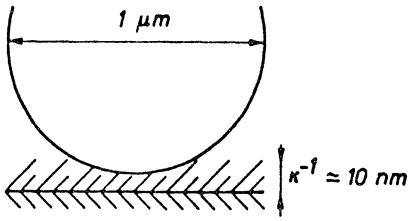


Figure 2. Schematic representation of a microbial cell attached to a surface which develops a diffuse double layer about 10 nm thick; note differences of scale.

In conclusion, while the surface electric potential of a support plays an important role for cell attachment (Rouxhet *et al.*, 1987; Mozes *et al.*, 1987, Mozes and Rouxhet, 1992), it is not

expected to influence greatly the cell activity. Note that the situation is very different when polyelectrolytes are brought in contact with cells; polycations such as chitosan can for instance penetrate the yeast cell wall and alter the membrane, giving rise to cell permeabilization (Champluvier *et al.*, 1988). The case in which the cells would be coated by a dense layer of inert but charged colloidal particles represents an intermediate situation.

3.3. SURFACE AND SHEAR FORCES

Hydrodynamic stresses exerted by the liquid may influence activity of individual cells or behaviour of a whole population of cells (such as a biofilm). Asther *et al.* (1990) investigated the basidiomycete *Phanerochaete chrysosporium* in view of production of a lignin peroxidase. The presence of certain supports (polypropylene rings, polyurethane foam cubes) led to an increased mycelium production, most of which was immobilized, and to an increased enzyme yield. Cell protection from the agitation seems to be responsible for the improved cell growth. However, the specific enzyme production (amount of enzyme produced per unit biomass) was lower for immobilized mycelium, as compared with pellets in suspension; this could possibly be due to limitation of oxygen supply to the immobilized cells.

3.4. INTERFACE AS A SPECIFIC ENVIRONMENT

Exopolymers produced by suspended bacteria may dissolve in water. However, the same macromolecules produced by bacteria at a solid surface may adsorb there. This will consolidate the bacterial adhesion and create a specific micro-environment. Water activity, viscosity, concentrations of low molecular weight solutes, diffusion rates, *etc.* will be changed within the gel-like matrix, and, as a consequence, the cell physiology will be affected. Thus the cellular activity of adherent bacteria will be influenced indirecly by the surface to which they adhere. Retention of water by polysaccharides may also protect bacteria from desiccation.

4. Other Factors Influencing the Activity of Immobilized Cells

Immobilization may influence the physiological state and the performance of cells through a number of parameters which may act in opposite directions. The confinement inherent to cell immobilization is such that the real environment of the biocatalyst (the micro-environment) is different from the bulk, or circulating, solution (the macro-environment), the composition of which is easily monitored and controlled. The literature is rich with conflicting reports concerning the effects of immobilization on microbial activity: increased activity (Navarro and Durand, 1977; Fletcher, 1986), decreased activity (Morikawa *et al.*, 1979), unchanged activity (Mozes and Rouxhet, 1984, 1985; Champluvier *et al.*, 1988), change of the ratio between structural and storage carbohydrates (Doran and Bailey, 1986), shift of optimal pH (Hattori and Furusaka, 1960), inhibition of cell division (Lindsy and Yeoman, 1984), increased plasmid stability and morphological modifications (Barbotin *et al.*, 1990). Most, if not all, of these examples represent actually indirect effects.

In the cases of immobilization in polymer beads, granules, or biofilms, the diffusion dependent mass transfer may be a limiting factor. For yeast cells entrapped in a silica hydrogel, calculations based on oxygen solubility and on its rate of uptake by the cells, indicate that only about ten cellular layers are active (Rouxhet *et al.*, 1981). Wheatley and Philips (1983) have shown the influence of internal and external diffusional limitations on the kinetics of *Alcaligenese*

faecalis cells immobilized in polyacrylamide gel. In the case of adhesion to a support as a monolayer, the convective mass transfer is much more efficient and no limitations are expected; such a system is suitable for studying the direct influence of the carrier surface on the cell activity.

4.1. INFLUENCE OF PROCEDURES USED FOR HANDLING CELLS

The conversion of glucose to ethanol by *Saccharomyces cerevisiae* immobilized by adhesion as a monolayer to glass plates was described by Mozes and Rouxhet (1985). Table 1 summarizes the initial rates of ethanol production for the immobilized cells and for the controls corresponding to the various treatments applied in order to promote adhesion. It shows that the activity of yeast immobilized after treatment with cations was lower than that of free non-treated cells. However, the loss of activity occurred mostly as a result of the pretreatment (low pH, contact with cations) and only to a limited extent as a result of immobilization. The activity of the cells immobilized on glass coated by colloidal particles was only 25% lower than that measured on non-treated free cells. Comparison between immobilized pretreated yeast and yeast attached directly to coated glass indicates that the decrease in activity is mainly due to handling the cells in physiologically unsuitable solutions. There is no significant evidence that cell fixation to the support, as such, influences their activity.

Table 1: *Saccharomyces cerevisiae* immobilized by adhesion as a monolayer to glass plates (Mozes and Rouxhet, 1985).

Means of achieving a monolayer	Initial ethanol production rate (10^{-17} mol cell^{-1} s^{-1})			
	Immobilized cells	Free cells		
		not treated	treated by M^+	treated by HNO_3
-	-	1.92	-	-
cells pretreated by Al^{3+} at pH 4	1.11	-	1.24	1.69
cells pretreated by Fe^{3+} at pH 3	0.97	-	1.22	1.47
support coated by $Al(OH)_3$	1.42	-	-	-
support coated by Fe_2O_3	1.39	-	-	-

4.2. TOXICITY OF SUBSTANCES RELEASED BY THE CARRIER

Figure 3 demonstrates the results of control experiments for pretreatment of yeast by Al^{3+} ions, which were carried out in order to test the toxicity of the cation (Rouxhet and Mozes, 1990). Loss of activity was moderate in the concentration range tested.

Pretreatment of yeast cells by colloidal particles of aluminium hydroxide (alumina), which had an amorphous character (Kayem and Rouxhet, 1983), led to flocculation and caused a lower production of ethanol (lower right corner of Figure 3); however, crystaline γ-AlOOH (Boehmite) did not affect the yeast activity (Mozes and Rouxhet, 1991). The metabolizing cells excrete

protons and, at pH 4, the solubility of $Al(OH)_3$ is already 2 10^{-2}M. The low activity of cells treated by amorphous alumina, may thus be the result of high local concentration of Al^{3+} ions. The crystaline boehmite is probably less susceptible to dissolution at low pH, compared to amorphous alumina.

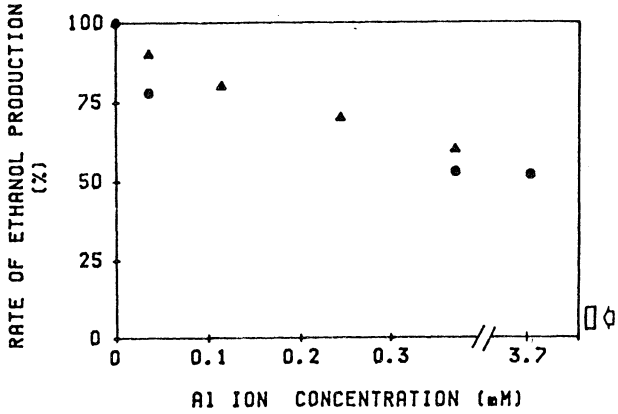


Figure 3. Relative variation of the rate of ethanol production by *Saccharomyces cerevisiae* suspension (5 g/L glucose in distilled water) as a function of the aluminium ions concentration to which the cells were exposed (cell suspension in $Al(NO_3)_3$ solution set at pH 4 by HNO_3, stirred for 1 h; cells centrifuged and washed by distilled water); the different signs are related to different sets of experiments. The rectangle indicated by the arrow in the lower right corner shows the range of activity measured when the cells are treated by $Al(OH)_3$ particles in distilled water (Rouxhet and Mozes, 1990).

The ethanol produced by *Saccharomyces cerevisiae* cells adhering to polystyrene (PS) grafted with amines was studied (Rouxhet and Mozes, 1990; Mozes and Rouxhet, 1991). It seemed that the tri-ethanolamine grafted PS had no, or only minor, influence on the ethanol production, while the yeast immobilized on tri-ethylamine grafted PS suffered severe reduction of activity. As a control, the effect of the free amines on the activity of freely suspended cells was also monitored. The production rates are given in Table 2 which includes also the initial and final pH of the tests with suspended cells. It is clear that the tri-ethylamine is toxic and the loss of activity of the cells attached to PS grafted with tri-ethylamine may be attributed to the toxicity of free amine released by the polymer. Surface analysis of the latter indicated that up to 30% of tri-ethylamine present in the polymer might be retained physically and not bound covalently. The total release of this amount into the solution used for testing the activity would give a concentration of about 0.03M tri-ethylamine. The severe loss of activity observed upon immobilization might be due to the existence of even higher concentration of the amine (progressively released into the solution) near the surface of the polymer which is the yeast micro-environment.

5. Conclusion

Mass transfer limitations are of prime importance for understanding the influence of immobilization on microbial cells, not only when the cells are immobilized in polymer beads, granules or biofilms, but also when particles, carrying cells attached on their surface, are piled up.

Table 2: *Saccharomyces cerevisiae* immobilized by adhesion to amine grafted polystyrene or suspended in the indicated solution (Rouxhet and Mozes, 1990).

	Initial ethanol production rate (10^{-17} mol cell^{-1} s^{-1})	pH initial	pH final
Immobilized cells			
PS grafted by :			
Triethanolamine	2.0		
Triethylamine	0.1		
Free cells			
suspension containing :			
no additive	2.3	4.1	3.4
NaOH	2.2	7.1	6.7
Triethanolamine (0.14M)	2.0	6.5	6.2
Triethylamine (0.03M)	1.1	5.9	5.5
Triethylamine (0.14M)	0	8.1	8.0

A carrier surface may release substances, eventually toxic, which are present in high concentrations in the cell micro-environment. The electric potential exerted by a charged support and responsible for a particular pH in the close vicinity of its surface is not expected to affect the activity of a microbial cell, the dimension of which is much larger than the thickness of the diffuse double layer. The vicinity of a surface may protect a micro-organism from shear forces. Adsorption of a nutrient by a solid carrier decreases its concentration in the solution to which the cells are exposed. On the other hand, direct transfer of the nutrient accumulated at the surface to the cell should be of negligible importance. The beneficial influence of a surface acting as a nutrient reservoir seems to be restricted to specific situations, for example starving conditions. The influence of a surface on production of cell appendages by certain bacteria is an example of a direct surface effect, which might act through local restriction of movement.

Acknowledgement

The authors' laboratory is affiliated with the Faculty of Agricultural Sciences; P.G.R. is a member of the Research Center for Advanced Materials. The Departement of Education and Scientific Research (Concerted Action of Physical Chemistry of Interfaces in Biotechnology), and the European Community (Biomolecular Engineering Programme and Biotechnology Action Programme) financed some of the works described in this review.

References

Asther, M., Bellon-Fontaine, M.N., Capdeville, C. and Corrieu, G. (1990), 'A thermodynamic model to predict *Phanerochaete chrysosporium* INA-12 adhesion to various solid carriers in relation to lignin peroxidase production', Biotechnol. Bioeng., **35**, 477-482.

Barbotin, J.N., Nava Saucedo, J.E. and Thomasset, B. (1990), 'Morphological observations on immobilized cells', in Physiology of Immobilized Cells (J.A.M. de Bont, J. Visser, B. Mattiasson and J. Trampers, eds.), Proc. Int. Symp. Wageningen, The Netherlands, 10-13 December 1989, Elsevier Science Publishers, Amsterdam, pp. 487-493.

Burchard, R.P. (1981), 'Gliding motility of prokaryotes: Ultrastructure, physiology, and genetics', Ann. Rev. Microbiol., **35**, 497-529.

Champluvier, B., Kamp, B. and Rouxhet, P.G. (1988), 'Immobilization of β-galactosidase retained in yeast: adhesion of the cells on a support', Appl. Microbiol. Biotechnol., **27**, 464-469.

Dagostino, L., Goodman, A.E. and Marshall, K.C. (1991), 'Physiological responses induced in bacteria adhering to surfaces', Biofouling, **4**, 113-119.

Dazzo, F.B. (1984), 'Bacterial adhesion to plant root surfaces', in Microbial Adhesion and Aggregation (K.C. Marshall, ed.), Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, pp. 85-93.

Doran, P.M. and Bailey, J.E. (1986), 'Effects of immobilization on growth, fermentation properties, and macromolecular composition of *Saccharomyces cerevisiae* attached to gelatin', Biotechnol. Bioeng., **38**, 73-87.

Durand, G. and Navarro, J.M. (1978), 'Immobilized microbial cells', Process Biochemistry, September 1978, 14-23.

Ellwood, D.C., Keevil, C.W., Marsh, P.D., Brown, C.M. and Wardell, J.N. (1982), 'Surface-associated growth' Phil. Trans. Roy. Soc. Lond., **B 297**, 517-532.

Fletcher, M. (1986), 'Measurement of glucose utilization by *Pseudomonas fluorescens* that are free-living and that are attached to surfaces', App. Environ. Microbiol., **52**, 672-676.

Fletcher, M. (1992), 'Bacterial metabolism in biofilms', in Biofilms: Science and Technology, Melo et al. (eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, this volume.

Hall, P.G. and Krieg, N.R. (1983), 'Swarming of *Azospirillum brasilense* on solid media', Can. J. Microbiol., **29**, 1592-1594.

Hattori, R. and Hattori, T. (1985), 'Adsorptive phenomena involving bacterial cells and an anion exchange resin', J. Gen. Appl. Microbiol., **31**, 147-163.

Hattori, R. and Hattori, T. (1987), 'Interaction of microorganisms with a charged surface - A model experiment', Reports of the Inst. for Agricultural Res., Tohoku Univ., **36**, 21-67.

Hattori, T. and Furusaka, C. (1960), 'Chemical activities of E. coli adsorbed on a resin', J. Biochem., **48**, 831-837.

Humphrey, B.A. and Marshall, K.C. (1984), 'The triggering effect of surfaces and surfactants on heat output, oxygen consumption and size reduction of a starving marine *Vibrio*', Arch. Microbiol., **140**, 166-170.

Humphrey, B.A., Dickson, M.R. and Marshall, K.C. (1979), 'Physicochemical and in situ observations on the adhesion of gliding bacteria to surfaces', Arch. Microbiol., **120**, 231-238.

Karel, S.F., Salmon, P.M., Stewart, P.S. and Robertson, C.R. (1990), 'Reaction and diffusion in immobilized cells: Fact and fantasy', in Physiology of Immobilized Cells (J.A.M. de Bont, J. Visser, B. Mattiasson and J. Trampers, eds.), Proc. Int. Symp. Wageningen, The Netherlands, 10-13 December 1989, Elsevier Science Publishers, Amsterdam, pp. 115-126.

Kayem G.J. and Rouxhet, P.G. (1983), 'Adsorption of colloidal hydrous alumina on yeast cells', J. Chem. Soc., Faraday Trans., **79**, 561-569.

Kefford, B., Humphrey, B.A. and Marshall, K.C. (1986), 'Adhesion: a possible survival strategy for Leptospires under starvation conditions', Current Microbiol., **13**, 247-250.

Kjelleberg, S., Humphrey, B.A. and Marshall, K.C. (1982), 'Effect of interfaces on small, starved marine bacteria', App. Environ. Microbiol., **43**, 1166-1172.

Kjelleberg, S., Humphrey, B.A. and Marshall, K.C. (1983), 'Initial phases of starvation and activity of bacteria at surfaces', App. Environ. Microbiol., **46**., 978-984.

Klein, J. and Ziehr, H. (1990), 'Immobilization of microbial cells by adsorption', J. Biotechnol., **16**, 1-16.

Lindsey, K. and Yeoman, M.M. (1984), 'The synthetic potential of immobilized cells of *Capsicum frutescens* Mill cv. annuum', Planta, **162**, 495-501.

Mattiasson, B. and Hahn-Hägerdal, B. (1982), 'Microenvironmental effects on metabolic behaviour of immobilized cells. A hypothesis.', Eur. J. Appl. Microbiol. Biotechnol., **16**, 52-55.

Morikawa, Y., Ochiai, K., Karube, I. and Suzuki, S. (1979), 'Bacitracin production by whole cells immobilized in polyacrylamide gel', Antimicrobial Agents and Chemotherapy, **15**, 126-130.

Morisaki, H. (1983), 'Effect of solid liquid interface on metabolic activity of *Escherichia coli*', J. Gen. Appl. Microbiol., **29**, 195-204 (1983).

Mozes, N. and Rouxhet, P.G. (1984), 'Dehydrogenation of cortisol by *Arthrobacter simplex* immobilized as supported monolayer', Enzyme Microb. Technol., **6**, 497-502.

Mozes, N. and Rouxhet, P.G. (1985), 'Metabolic activity of yeast immobilized as supported monolayer', Appl. Microbial Biotechnol., **22**, 92-97.

Mozes, N. and Rouxhet, P.G. (1991), 'Effects of the micro-environment on metabolic activity of immobilized yeast cells', Mededelingen van de Faculteit Landbouwwetenschappen, Rijksuniversity Gent , **56**, 1761-1768.

Mozes, N. and Rouxhet, P.G. (1992), 'Modification of surfaces for promoting cell immobilization' in Biofilms: Science and Technology, Melo et al. (eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, this volume.

Mozes, N., Marchal, F., Hermesse, M.P., Van Haecht, J.L., Reuliaux, L., Léonard, A.J. and Rouxhet, P.G. (1987), 'Immobilization of microorganisms by adhesion: Interplay of electrostatic and nonelectrostatic interactions', Biotechnol. Bioeng., **30**, 439-450.

Navarro, J.M. and Durand, G. (1977), 'Modification of yeast metabolism by immobilization onto porous glass', European J. Appl. Microbiol., **4**, 243-254.

Rouxhet, P.G. (1991), 'Interfacial interactions with microorganisms: Application in fermentation technology', Biofouling, **4**, 151-161.

Rouxhet, P.G. and Mozes, N. (1990), 'The micro-environment of immobilized cells: critical assessment of the influence of surfaces and local concentrations', in Physiology of Immobilized Cells (J.A.M. de Bont, J. Visser, B. Mattiasson and J. Trampers, eds.), Proc. Int. Symp. Wageningen, The Netherlands, 10-13 December 1989, Elsevier Science Publishers, Amsterdam, pp. 343-354.

Rouxhet, P.G., Mozes, N., Hermesse, M.P. and Matta-Ammouri, G. (1987), 'Immobilization of microorganisms by adhesion to a support: influence of physico-chemical factors', in Proc. 4th European Congress on Biotechnol. (O.M. Neijssel, R.R. van der Meer and K.Ch.A.M. Luyben, eds.), Elsevier Science Publishers, Amsterdam, pp. 193-196.

Rouxhet, P.G., Van Haecht, J.L., Didelez, J., Gerard, P. and Briquet, M. (1981), 'Immobilization of yeast cells by entrapment and adhesion using siliceous materials', Enzyme Microb. Technol., **3**, 49-54.

Silverman, M., Belas, R. and Simon, M. (1984), 'Genetic control of bacterial adhesion', in Microbial Adhesion and Aggregation (K.C. Marshall, ed.), Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, pp. 95-107.

Sorongon, M.L., Bloodgood, R.A. and Burchard, R.P. (1991), 'Hydrophobicity, adhesion and surface-exposed proteins of gliding bacteria', Appl. Environm. Microbiol., **57**, 3193-3199.

Van Loosdrecht, M.C.M., Lyklema, J., Norde, W. and Zehnder, A.J.B. (1990), 'Influence of interfaces on microbial activity', Microbiol. Rev., **54**, 75-87.

Wheatley, M.A., Phillips, C.R. (1983), 'The influence of internal and external diffusional limitations on the observed kinetics of immobilized whole bacterial cells with cell-associated ß-glucosidase activity', Biotechnol. Letters, **5**, 79-84.

EXTRACELLULAR POLYMERS IN BIOFILMS

K.E. Cooksey
Montana State University
Bozeman, MT 59717
U.S.A.

1. Introduction

Many of those who study biofilms view them as a collection of living organisms at an interface but this definition should be expanded to include the products of those organisms. A major product is the matrix in which biofilm cells are found. It is somewhat surprising that there is such an emphasis on the biotic component of the film because this phase occupies only a small fraction of the volume (Characklis & Cooksey, 1983). It is often the biofilm matrix that causes many of the economic problems associated with biofilm formation since it acts as a layer of immobilized water. It is in fact highly hydrated and contains 98-99% water (Christensen and Characklis, 1990). This matrix, which is really a collection of polymers rather than a single material, is made by many organisms in biofilms. The polymers have been referred to collectively as capsules, sheaths, slime and glycocalyces. Costerton *et al* (1981) proposed the term glycocalyx for use in procaryotic biology. They defined a glycocalyx as "those polysaccharide-containing structures of bacterial origin, lying outside the integral elements of the outer membrane of Gram-negative cells and the peptidoglycan of Gram-positive cells". They further subdivided glycocalyces into (1) glycoprotein subunits at the cell surface and (2) capsules. "Capsules" were further subdivided into (a) those that are rigid and exclude particles such as Indian ink (a classical negative "stain" in bacteriology); (b) those, which in contrast to (a), are flexible and include Indian ink; (c) integral capsules that are closely associated with the cell surface and (d) those capsules that are peripheral to the cell and can be lost to the aqueous phase. In a brief but comprehensive review (Geesey, 1982), Geesey used a less structured term for the high molecular weight material extracellular to cells. He referred simply to extracellular polymeric substances and included all types of cells, not

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 137–147.

just bacteria. Although not the first to use it, this review is largely responsible for the general use of the term now universally abbreviated as EPS. Because of its ease of use (e.g. it requires no knowledge of chemical structure), I believe the term EPS has come to be regarded almost as a substance in its own right, rather than a collective term used to describe a poorly-understood group of macromolecules, external to the cell and of differing structure.

In this article I will use the term EPS as described by Geesey rather than by Costerton, since I wish to include all organisms at surfaces. In 1983, Characklis & Cooksey reviewed the role of EPS in biofilm formation and summarized their ideas speculatively as follows: EPS "may (1) provide cohesive forces within the biofilm, (2) adsorb nutrients, (3) protect immobilized cells from environmental changes, including the influence of biocides, (4) adsorb heavy metals from the environment, (5) adsorb particulate and other detritus, (6) serve as a means of intercellular communication within the biofilm, (7) provide short-term energy storage via the cell membrane potential and (8) enhance intercellular transfer of genetic material. Although a great deal of progress has been made since, these thoughts, in some cases, must still be treated as speculation, in spite of the research effort over the intervening years (e.g. Dahlem conferences, 1984, 1989).

Although many organisms in a climax biofilm contribute to the formation of EPS (Marzalek *et al*, 1979) only those of bacteria and microalgae will be reviewed here.

2. Bacterial EPS

2.1. COMPOSITION

It should be mentioned at the outset that no component of the EPS known to be implicated in the adhesive process has been comprehensively analyzed. Rather we have a series of fragmented studies addressing only part of the problem.

As mentioned in the introduction, many workers have prepared a polysaccharide material from the medium after the growth of bacterial cells. A procedure relying heavily on dialysis to remove low M.W. growth medium components, followed by ethanolic precipitation (80-90% v.v.) is usually used. There have rarely been attempts to discover the number of macromolecules present. Yet in 1973 Fletcher & Floodgate proposed the involvement of two polysaccharides in the adhesive process of *Pseudomonas* strain NCMB 2021. The first of these was thought to be involved in the initial adhesion and was presumed to exist on the surface of all cells. The second polymer was produced subsequent to the adhesive event and possibly caused a firmer association with the substratum. An alternative explanation is that the secondary polymer is produced only by cells in the "resting phase" - a state sometimes applied to cells adhered in biofilms. Cations, especially calcium, are important in maintaining the structure. Working with the same organism, Christensen *et al* (1985) have published the most detailed information to date on EPS conformation, but even this does not go as far as a primary structure for the polymers found. These workers found that *Pseudomonas* strain NCMB 2021

produces two very dissimilar polysaccharides. The first is produced only in the logarithmic phase of growth and contains the sugars glucose, galactose, glucuronic acid and galacturonic acid. It produces a viscous solution. The second polymer is produced in the stationary phase and contains N-acetylglucosamine, 2-keto-3-deoxyoctulosonic acid and an unidentified 6-deoxyhexose. A solution of this hydrophobic polymer is not viscous and is soluble in 80% (v/v) ethanol. The production of this polymer is closely correlated with the ability of the organism to attach to hydrophobic polystyrene. The polymers were separated by chromatography on Sepharose CL-2B and DEAE-Sephadex. The acid stability of each was different and this too allowed distinction to be made between them. Even though very complete, the analyses accounted for only 71% of the starting material. This may have been, the authors suggest, a problem with the hydrolysis of polymers containing uronic acids. Such groups are known to stabilize glycosidic bonds in carbohydrates and reduce the yield of hydrolytic products (Sharon, 1975).

As a result of NMR analysis, the authors thought that the stationary phase polymer contained a trisaccharide repeating unit with 0.95 units of 0-acetyl groups per unit. This polymer would not have been detected in any of the studies presented below since all of them depend on a high concentration of an alcohol to precipitate the EPS prior to analysis. Before leaving the work of Christensen *et al*, it is tempting to suggest that the two polymers described were identical to those mentioned by Fletcher & Floodgate. Uhlinger & White (1983) reported a variation in the galactose content of *Pseudomonas atlantica* over the growth cycle. It is suggested that since they did not endeavor to separate individual polymers from the EPS, it is likely that they were analyzing at least two polymers whose individual concentrations in the medium were growth phase-dependent.

Sutherland (1980) prepared polysaccharides from medium after the growth of uncharacterized marine and freshwater bacteria. The EPS was precipitated with acetone or isopropanol. The sugars found were mannose, glucose and galactose predominantly with galacturonic and glucuronic acids. Usually only two sugars were present in one preparation which was assumed to be a single polymer. Ford *et al* (1991) used pyrolysis mass spectrometry to survey a similar set of bacteria to those of Sutherland. Again, a single polymer was assumed. The pyrolysis fragments found may be indicative of differences between the polymers of individual species. Very little protein was found in the polymers and only 27-45% carbohydrate, of which a small fraction was uronic acids. The authors recognize that their technique at its present stage of development produces only a "finger-print" analysis. However, if the finger-prints for the EPS of various organisms are sufficiently distinct, the technique could be used in survey work. Only a few micrograms of the polymer are needed.

Plude *et al* (1991) also assumed that *Microcystis flos-aquae* formed only one kind of EPS. The polymer, which was water-soluble, bound iron and calcium very strongly. Its sugar components were very similar to those of higher plant pectin (galacturonic acid / rhamnose / mannose / xylose / glucose / galactose = 43/3/3/2/1/1). The proportion of galacturonate was extremely high for a microbial EPS. An interesting note in their paper is that they used a sugar mixture to standardize the anthrone reaction for carbohydrate

determination, not a single sugar as is more usual.

Humphrey *et al* (1979) grew a *Flexibacter* sp. on a dialysis membrane since no detectible EPS was synthesized in suspended culture. The alcohol precipitated EPS was comprised of glucose, fucose, galactose and some uronic acids. The most notable aspect of this EPS was that it contained 30% protein. The polymer, which was highly hydrophobic, was a glycoprotein. Humphrey *et al* note that *Flexibacteria* move by gliding and thus need to adhere to a surface by Stefan, not a permanent type adhesive. Is this reflected in the fact that this flexibacterial EPS is a glycoprotein, whereas more permanent bacterial adhesives are acid polysaccharides?

The interest of Abu *et al* (1991) in the EPS of *Shewanella colwelliana* stems from its involvement in the settlement of oyster spat. The production of EPS was enhanced when the organism was grown on the surface of a dialysis membrane (8000 M.W. cut off) lying on marine agar. The ethanol precipitated EPS was found to have a broad peak on a gel filtration column but multiple peaks on DEAE-cellulose. Gas chromatography-mass spectrographic analysis (GCMS) showed the presence of a hydrocarbon component. No hydrolytic sugar analysis was performed, but the semipurified polymer contained only 15-35% carbohydrate of which a large fraction was glucose (GCMS).

The physical structure of EPS can be seen in the electron microscope, but only after a dehydration step that causes considerable artefact. This is not surprising since EPS in a film is 99% water. Attempts to overcome this have been made with polycationic heavy metal stains such as ruthenium red. Even with ruthenium red staining EPS collapses, but a least the condensed material can be seen. It is highly likely that ruthenium red will stain acid proteoglycans and so should not be considered specific for acid polysaccharides (Handley, 1991).

Costerton's laboratory has long recommended the use of EPS-specific antibodies to stabilize material dehydrated for both transmission and scanning electron microscopy. Judging by the micrographs from this laboratory, the technique is very successful (Costerton, 1986). Probably because of the time and effort required to prepare specific antibodies, this technique is used by few laboratories, however.

In the last few years, workers in the field of EPS research have begun to question whether the polymers prepared from media in which adhesive cells have grown are the ones involved in adhesion. Further, they are questioning whether the polymer involved in the initial adhesive event is identical to that found as the matrix in a mature single species biofilm. Marshall *et al* (1989) designed physicochemical experiments to discern whether two such polymers exist in *Pseudomonas fluorescens*. They used two light microscope techniques. In the first of these, interference reflectance microscopy (IRM) (Curtis, 1964), the image of a cell becomes darker as it approaches the substratum (range of measurement = 0-100 nanometers). The second technique, light sectioning microscopy (LSM) (Loeb, 1980) operates at a greater separation distance (10-1000 micrometers) and is capable of measuring total film thickness. An example of the initial adhesive film was that elaborated by the organism immediately on attaching to a glass cover slip; a three day old surface culture was used to exemplify a biofilm containing matrix polymer. When viewed in the IRM or LSM, various agents caused contraction

(calcium or lanthanum ions) or expansion (Tween 20) of both the initial adhesive polymer and the biofilm matrix polymer. There was a differential response with dimethyl sulfoxide however (DMSO). The initial adhesive polymer contracted but the matrix polymer was unaffected. In general, the two polymers appeared to be physicochemically similar.

Allison & Sutherland (1987) also question whether the effort expended in determining the properties of EPS isolated from media is misplaced. They measure total EPS production with a specific carbohydrate binding dye (congo red) in two organisms. One was a wild type, manufactured EPS, was adhesive and formed mucoid colonies. A mutant that did not make EPS adhered as well as the wild-type organism, but it did not make colonies. Thus Allison & Sutherland propose that the EPS formed by the wild-type organism is involved in biofilm matrix formation, but not in the initial adhesion of the cells.

Taking this argument one step further, Neu & Marshall (1991) suggest that the material left behind when bacteria are removed from a surface is really the cellular adhesive and it is on this polymer that we should concentrate our efforts. This has also been proposed by Cooksey & Cooksey (1986). It follows that the EPS found in the growth medium and that left behind as "footprints" should be compared.

2.2. SYNTHESIS

EPS synthesis is somewhat difficult to describe given the fact that more than one polymer appears to be present and these are likely to have differing composition. The general biochemistry of extracellular carbohydrate synthesis is reviewed by Sutherland (1982). Other work that will not be considered here is that dependent on measurement of the production of EPS under varying conditions where the analyses were carried out by differential analyses of carbon in the effluent from a reactor (i.e. total carbon - cellular carbon = EPS carbon). These studies (Bakke *et al*, 1984; Robinson *et al*, 1984) although informative in a stoichiometric modeling sense, give only anecdotal information concerning the biochemical and genetic controls of the cell. *In situ* determination of biofilm EPS is of some interest however. Bremer & Geesey (1991) described a dual flow system where protein and carbohydrate (EPS) in a biofilm could be measured quickly and non-destructively. By comparing the Fourier Transform Infra Red (FTIR) spectra of two flow cells, one inoculated with bacteria and the other sterile, the accumulation of cellular protein and matrix EPS could be measured with time. The uninoculated cell provided a blank signal. Only the film was sampled by the infra-red beam since beam penetration was less than one bacterial cell in depth. Bremer & Geesey found that the EPS increased along with protein for 75h and then was constant for the next 100h. This suggests that the ratio of cells to EPS at the interface of a biofilm and the substratum is constant.

Wrangstadh *et al* (1990) demonstrated that *Pseudomonas* sp. Strain S9 produced two forms of EPS. The first of these was closely associated with the cell during growth (integral EPS) and the second (peripheral EPS), more loosely so. The first polymer was

produced during growth and both were produced during starvation conditions. These polymers appear rather similar to those described by Fletcher & Floodgate (1973) for *Pseudomonas* strain NCMB 2021. Wrangstadh *et al* concluded that the formation of the peripheral EPS was a response to starvation. If protein synthesis was inhibited with chloramphenicol at the onset of starvation, no formation of peripheral EPS took place. Thus proteins induced by starvation conditions were responsible for its synthesis, or its production from shorter chain precursors. This, and other results presented in the paper, demonstrated that the integral form of the EPS, but not the peripheral form, promoted adhesion.

It is a frequent observation that bacterial cells that produce EPS do so to a greater extent on solid medium than in liquid culture (e.g. Sutherland, 1980; Abu *et al*, 1991). I believe that these are not anecdotal observations. Schneider et al (1991) demonstrated that a non-mucoid strain of *P. aeruginosa*, when growing on a dialysis membrane placed on an agar surface, showed sensitivity to the MW cut off of the membrane by controlling the amount of EPS produced. Where the dialysis membrane allowed diffusion of compounds of molecular weight 30-50KDa, no EPS was produced. A membrane with a lower molecular weight cut-off allowed slime synthesis. This suggests that lack of diffusion of the range of molecules (30-50KDa) influences polymer production on a surface and therefore may act as a signalling mechanism that indeed the cell is on a surface. These observations fit well with those of Wrangstadh *et al* (1990). The mechanism for cell-surface sensing has been proposed previously for diatoms (Wigglesworth-Cooksey & Cooksey, 1992).

2.3. BREAKDOWN OF BACTERIAL EPS.

Geesey (1982) has suggested that EPS is not broken down as a carbon source by the cells that were responsible for its synthesis. Rodrigues and Bhosle (1991) observe that EPS in a *Vibrio fischeri* biofilm does not decrease with biofilm age.

3. Microalgae

This discussion will be restricted to the EPS of diatoms since these organisms form the main microalgal component of illuminated biofilms in both freshwater and marine environments. Some of the general remarks made concerning bacterial EPS, also apply to microalgal EPS (e.g. staining, water content).

3.1. COMPOSITION

A large number of diatom species have extracellular structures composed wholly or partially of carbohydrate. These include stalks, tubes of tube-dwelling diatoms and slime of various kinds. The extracellular slime includes the so-called trail substance of motile pennate diatoms. The sugar composition of earlier analyses has been reviewed previously (Characklis & Cooksey, 1983; in Werner, 1977; and in Stewart, 1974). As in the studies on bacteria, most, if not all, analyses have been performed with the

assumption that the analyte is a single macromolecular carbohydrate. Examples of such analyses showed the presence of: mannose, rhamnose and galactose in *Nitzschia frustulum* (Allan, 1972); xylose, mannose, fucose and galactose in *Phaeodactylum tricornutum* (Lewin, 1958) and mannose, glucuronic acid, glucose, galactose rhamnose, xylose and fucose in *Navicula pelliculosa* (Coombs & Volcani, 1968), i.e. constituents not dissimilar to those in bacterial EPS. Although some linkage work has been carried out, it is of limited use at present because in no case so far were the polymers shown to be a single compound before hydrolysis.

Staining of the unpurified polymers has shown them to be sulphated acid polysaccharides (Chamberlain, 1976; Daniel *et al*, 1987).

3.2. SYNTHESIS OF DIATOM EPS

Although only a few organisms have been studied, it is likely that they do not vary greatly in their synthetic processes. In *Amphora*, an acid polysaccharide synthesized in the Golgi apparatus is packaged into vesicles which are transported to raphe opening where they fuse with the plasmamembrane before releasing their contents into the extracellular space (Daniel *et al*, 1980; Webster *et al*, 1985; Edgar & Pickett-Heaps, 1983). Webster *et al* showed EPS material secreted along the entire length of the raphe. In a series of papers from this laboratory on *Amphora coffeaeformis* (Cooksey, 1981; Cooksey & Cooksey, 1986; Cooksey & Cooksey, 1988, Webster *et al*, 1985) it was shown that the result of EPS secretion, i.e. motility and adhesion, was sensitive to inhibition by energy uncouplers, (carbonyl cyanide m-chlorophenyl hydrazone, pentachlorophenol), a protein synthesis inhibitor (cycloheximide), a glycoprotein synthesis inhibitor (tunicamycin) anticytoskeletal inhibitor (cytochalasins) but not inhibitors of photosynthesis (3^1(3,4-dichlorophenyl) $1,1^1$ dimethyl urea, darkness). The process was also shown to be calcium-dependent, with calcium being required internally and externally to the cell (inhibition by lanthanum and D-600, detachment caused by EGTA) (see model in Wigglesworth-Cooksey & Cooksey, 1992).

We have referred earlier to a hypothesis whereby continued secretion of small quantities of this polymer, even when cells are suspended in the water column, may allow a diatom cell to "sense" a surface when it alights because of restricted diffusion of the polymer (Section 2.2.). At the moment there is no means to differentiate between the EPS used for the initial adhesion, that secreted continuously as a trail substance or motility polymer for gliding locomotion and that secreted when cells become permanently adhered. The motility polymer is soluble in the aqueous medium (Webster et al, 1985). The permanent adhesive EPS can be prepared by removing cells from a surface with EGTA (Cooksey & Cooksey, 1986). Antibodies raised to either of these EPS will allow distinctions to be made. This is our current research plan.

4. Ecological Considerations

In assessing an ecological niche, scientists often and quite understandably, approach the situation from an anthropomorphic point of view. They consider the advantages to the organism of its occupancy of the particular ecological situation. Rarely do they believe that the organism occupies the niche by chance and its reactions are only those of survival under what may be adverse conditions. For instance, Characklis & Cooksey, (1983) suggested that "Perhaps the surface may be an inhospitable environment where an organism becomes trapped, and, as a means of survival produces an extracellular polymer ..... (which) .. effectively insulates the cell from the stresses of the environment". A great deal has been written concerning the advantages of the surface-attached state for microorganisms, however we are only now coming to an understanding that is not always the case (Fletcher, 1991). It is within this framework that the ecological role of EPS will be discussed.

There is no doubt that EPS provides a diffusion barrier to nutrients and cellular products (Cooksey, 1992). However, the degree to which diffusion is hampered is not great. For instance, Jensen & Revsbech (1989) showed that oxygen diffusion in a biofilm was only half that in free aqueous medium. Is this diminution sufficient to cause major physiological changes in the cells? The EPS in a biofilm has been considered to be involved in the concentration of nutrients from the overlying aqueous phase. EPS is usually an acid polysaccharide with carboxyl and sometimes sulfate groups. At the pH values that exist normally in nature (say pH6-8), this means that it could only complex with positively-charged ions. Very few microbial nutrients are positively-charged at these pH values. Even if positively charged nutrients were complexed, the affinity of microbial transmembrane transport systems would need to be greater than the binding to the matrix EPS.

The close proximity of cells in a biofilm may promote genetic exchange (Characklis & Cooksey, 1983). This idea has been developed by Stal *et al* (1989) and examples where this has happened have been provided. Microbial cells are more resistant to biocides when in films (Costerton & Marrie, 1983). The fact that such cells are embedded in EPS has been proposed as the reason. However, Nichols *et al* (1989) have refuted this idea, even for positively charged antibiotics such as tobramycin (Nichols *et al*, 1989).

The relative cohesive and adhesive strengths of microbial films on solid surfaces has been discussed earlier (Cooksey, 1992). It is not known whether the presence of multiple organisms in a film increases these strengths because of the physicochemical interactions of the various EPS produced by individual species (i.e. an increased gel strength by co-gellation), or whether the presence of one organism influences another adjacent organism to produce altered EPS that also has altered gelling properties.

Stal *et al* (1989) has stated that the reasons for EPS production are not completely understood. Christensen & Characklis (1990) have called EPS a "magic substance" - it is suggested that the word "mystic" is more appropriate!

5. Acknowledgements.

The financial support of the U.S. Office of Naval Research is acknowledged.

6. References.

Abu, G.O., Weiner, R.M. and Colwell, R.R. (1991) "Properties of an extracellular adhesive polymer from the marine bacterium *Shewanella colwelliana*", Biofouling, 3, 69-84.

Allison, D.G. and Sutherland, I.W. (1987) "The role of exopolysaccharides in adhesion of freshwater bacteria", J. Gen. Microbiol. 133, 1319-1327.

Bakke, R., Trulear, M.G., Robinson, J.A. and Characklis, W.G. (1984) "Activity of *Pseudomonas aeruginosa* in biofilms: Steady state. "Biotech. Bioeng. 26, 1418-1424.

Bremer, P.J. and Geesey, G.G. (1991) "An evaluation of biofilm development utilizing non destructive attenuated total reflectance Fourier transform infrared spectroscopy", Biofouling 3, 89-100.

Characklis, W.G. and Cooksey, K.E. (1983) "Biofilms and microbial fouling" in A.I. Laskin (ed.) Applied Microbiology, 29, Academic Press, New York, pp. 83-138.

Christensen, B.E. and Characklis, W.G. (1990) "Physical and chemical properties of biofilms" in W.G. Characklis and K.C. Marshall (eds.) Biofilms, Wiley-Interscience, New York, pp. 93-130.

Christensen, B.E. Kjosbakken, J. and Smidsrod, O. (1985) "Partial chemical and physical characterization of two extracellular polysaccharides produced by marine, periphytic *Pseudomonas* sp. Strain NCMB 2021". Appl. Environ. Microbiol. 50, 837-845.

Cooksey, K.E. (1992) "Bacterial and algal interactions in biofilms" in Melo et al, (eds.) Biofilms: Science and Technology, Kluwer Academic Press, Dordrecht, The Netherlands, this volume.

Cooksey, K.E. and Cooksey, B. (1986) "Adhesion of fouling diatoms to surfaces: Some biochemistry", in L.V. Evans and K.D. Hoagland (eds.) Algal Biofouling, Elsevier, Amsterdam, pp. 41-53.

Cooksey, K.E. and Wigglesworth-Cooksey, B. (1992) "The design of antifouling surfaces: Background and some approaches", in Melo et al, (eds.) Biofilms: Science & Technology, Kluwer Academic Publishers, Dordrecht, The Netherlands, this volume.

Coombs, J. and Volcani, B.E. (1968) "Studies on the biochemistry and fine structure of silica shell formation in diatoms. Chemical changes in the wall of *Navicula pelliculosa* during its formation". Planta 82, 280-292.

Costerton, J.W., Irvin, R.T. and Cheng, K.-J. (1981) "The bacterial glycocalyx in nature and disease", Annu. Rev. Microbiol. 35, 299-324.

Costerton, J.W. and Marrie, T.J. (1987) "The role of the bacterial glycocalyx in resistance to antimicrobial agents", in C.S.F. Easmon, J. Jelzaszewicz, M.R.W. Brown & P.A. Lambert (eds.), The Role of the Envelope in the Survival of Bacteria in Infections, Academic Press, London, pp. 63-85.

Costerton, J.W., Nickel, J.C. and Ladd, T.I. (1986) "Suitable methods for the comparative study of free-living and surface associated bacterial populations", in J.S. Poindexter and E.R. Leadbetter (eds.) Methods and Special Applications in Bacterial Ecology, 2, Plenum, N.Y. pp. 49-84.

Daniel, G.F., Chamberlain, A.H.L. and Jones, E.B.G. (1980) "Ultrastructural observations on the marine fouling diatom *Amphora*". Helgolander meeresunters. 34, 123-129.

Daniel, G.F., Chamberlain, A.H.L. and Jones, E.B.G. (1987) "Cytochemical and electron microscopical observations on the adhesive materials of marine fouling diatoms". British Phycol. J. 22, 101-118.

Edgar, L.A. and Pickett-Heaps, J. (1983) "The mechanism of diatom locomotion. I. An ultrastructural study of the motility apparatus". Proc. Roy. Soc. Lond. B 218, 331-343.

Fletcher, M. (1991) "The physiological activity of bacteria attached to solid surfaces", in A.H. Rose and D.W. Tempest (eds.) Advances in Microbial Physiology 32, Academic Press, London, pp. 53-85.

Fletcher, M. and Floodgate, G.D. (1973) "An electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces", J. Gen. Microbiol. 74, 325-334.

Ford, T., Sacco, E., Black, J., Kelley, T., Goodacre, R., Berkeley, R.C.W. and Mitchell, R. (1991) "Characterization of exopolymers of aquatic bacteria by pyrolysis-mass spectrometry", Appl. Environ. Microbiol. 57, 1595-1601.

Geesey, G.G. (1982) "Microbial exopolymers: Ecological and economic considerations", Amer. Soc. Microbiol. News, 48, 9-14.

Humphrey, B.A., Dickson, M.R. and Marshall, K.C. (1979) "Physicochemical and *in situ* observations on the adhesion of gliding bacteria to surfaces." Arch. Microbiol. 120, 231-238.

Jensen, J. and Revsbech, N.-P. (1989) "Photosynthesis and respiration of a diatom biofilm cultured in a new gradient growth chamber." FEMS Microbiol. Ecol. 62, 29-38.

Lewin, R.A. (1958) "The mucilage tubes of *Amphipleura urtilans*." Limnol. Oceanogr. 3, 111-113.

Loeb, G.I. (1980) "Measurement of microbial marine fouling films by light section microscopy." Mar. Tech. Soc. J. 14, 17-19.

Marshall, P.A., Loeb, G.I., Cowan, M.M. and Fletcher, M. (1989) "Response of microbial adhesives and biofilm matrix polymers to chemical treatment as determined by interference reflection microscopy and light section microscopy. Appl. Environ. Microbiol. 55, 2827-2831.

Marzalek, D.S., Gerchakov, S.M. and Udey, L.R. (1979) "Influence of substrate composition on marine microfouling." Appl. Environ. Microbiol. 38, 987-995.

Neu, T.R. and Marshall, K.C. (1991) "Microbial 'footprints'- A new approach to adhesive polymers." Biofouling 3, 101-112.

Nichols, W.W., Evans, M.J., Slack, M.P.E. and Walmsley, H.L. (1989) "The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*." J. Gen. Microbiol. 135, 1291-1303.

Plade, J.L., Parker, D.L., Schommer, O.J., Timmerman, R.J., Hagstrom, S.A., Joers, J.M. and Hnasko, R. (1991) "Chemical characterization of polysaccharide from the slime layer of cyanobacterial *Microcystis flos-aquae* C3-4D." Appl. Environ. Microbiol. 57, 1696-1700.

Robinson, J.A., Trulear, M.G. and Characklis, W.G. (1984) "Cellular reproduction and extracellular polymer formation by *Pseudomonas aeruginosa* in continuous culture." Biotech. Bioeng. 26, 1409-1417.

Rodrigues, C., and Bhosle, N.B. (1991) Expolysaccharide production by *Vibrio fischeri*, a fouling marine bacterium. Biofouling, 4, 301-308.

Schneider, H., Ross, N., and Levitan, R. (1991) "Surface-associated factors in the formation of biofilms: Effects of surface porosity on slime formation by *Pseudomonas aeruginosa, Staphylococcus aureas* and *Staphylococcus epidermis*", in J.A.M. de Bont, J. Visser, B. Mattiasson and J. Tramper (eds.), Physiology of Immobilized Cells, Elsevier, Amsterdam, pp. 99-102.

Sharon, N. (1975) "Complex carbohydrates. Their chemistry, biosynthesis, and functions." Addison-Wesley, Reading, MA. U.S.A.

Stal, L.J. (1989) "Cellular physiology and interactions of biofilm organisms" in W.G. Characklis and P.A. Wilderer (eds.), Structure and Function in BIofilms, Dahlem Conference Wiley Interscience, Chichester, pp. 269-286.

Stewart, W.D.P. (1974) "Algal physiology and biochemistry", W.D.P. Stewart (ed.) Botanical Monographs 10, University of California, pp. 989.

Sutherland, I.W. (1980) "Polysaccharides in the adhesion of marine and freshwater bacteria", in R.C.W. Berkeley, J.M. Lynch, J. Melling, P.O. Rutler and B. Vincent (eds.) Microbial Adhesion to Surfaces, Ellis Harwood, London, pp. 329-338.

Sutherland, I.W. (1982) "Biosynthesis of microbial exopolysaccharides" in A.H. Rose and J. G. Morris (eds.), Advances in Microbial Physiology, 23. Academic Press, London, pp. 79-150.

Uhlinger, D.J. and White, D.C. (1983) "Relationship between physiological status and formation of extracellular polysaccharide glycocalyx in *Pseudomonas atlantica*." Appl. Environ. Microbiol. 45, 64-70.

Webster, D.R., Cooksey, K.E. and Rubin, R.W. (1985) "An investigation of the involvement of cytoskeletal structures and secretion in gliding motility of the marine diatom *Amphora coffeaeformis*." Cell Motility 5, 103-122.

Werner, D. (1977) "The biology of diatoms." Botanical Monographs, University of California, pp. 498.

Wigglesworth-Cooksey, B. and Cooksey, K.E. (1992) "Can diatoms sense surfaces: State of our Knowledge." Biofouling, 5, 227-238.

Wrangstadh, M., Szewzyk, U., Ostling, J. and Kjelleberg, S. (1990) "Starvation-specific formation of a peripheral exopolysaccharide by a marine *Pseudomonas* sp., Strain S9." Appl. Environ, Microbiol. 56, 2065-2072.

FORMATION, COMPOSITION AND PHYSIOLOGY OF ALGAL BIOFILMS

B.S.C. LEADBEATER and MAUREEN E. CALLOW
School of Biological Sciences
The University of Birmingham,
Birmingham B15 2TT, United Kingdom

1. Introduction

Algal biofilms will develop on any illuminated surface which is either submerged in water or exposed provided there is moisture in the atmosphere and nutrients are available. In natural ecosystems, algal biofilms are familiar on the surface of stones in rivers and on the seashore or in the aerial environment on garden walls and the bark of trees. Algal biofilms on man made structures are best known for the nuisance they cause both in terms of safety and economics.
The most severe problems associated with algal fouling are on submerged structures particularly in the marine environment. Algal fouling occurs on static structures such as offshore oil and gas platforms, OTEC platforms, pipelines, buoys, pilings, piers, culverts, swimming pools, aquaculture tanks, fishing nets and on moving structures such as boats, ships and submarines. Efficient functioning of industrial plant apparatus such as heat exchanger tubes, plastic water pipes and cooling towers may also be impaired by algal biofilms. Algal biofilms also pose aesthetic problems by disfiguring building exteriors, fences, signposts and works of art such as statues.
In the aquatic environment, communities develop on unprotected man-made structures similar to those found on natural substrates such as rocks. In coastal waters, where the potential for colonization is enormous, a climax community might typically comprise large seaweeds such as kelps and a variety of soft and hard bodied invertebrates such as mussels and barnacles. Such climax communities are well documented for submerged static structures such as pilings, piers, and offshore oil and gas platforms where anti-fouling measures are only infrequently employed. The problems caused by climax communities of macrofouling organisms relate chiefly to increased drag and hydrodynamic loading. These aspects have been discussed previously in a number of comprehensive reviews (see e.g. Costlow & Tipper, 1984; Callow & Edyvean, 1990). The present article will be concerned with algae within biofilms which form compact coverings on surfaces (fig. 1). Although biofilms are typically composed of unicellular or simple filamentous organisms often referred to as microfouling or slimes, in certain circumstances the surrounding environment e.g. at high water flow velocities or in the presence of toxins, causes stunted growth forms e.g. macroalgae such as *Enteromorpha* and *Ectocarpus* growing on anti-fouling paints. Under such conditions, the algae can adopt a compact growth habit or become integrated into the microfouling slime layer. In this article the term algal biofilm is used in the broadest sense.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 149–162.

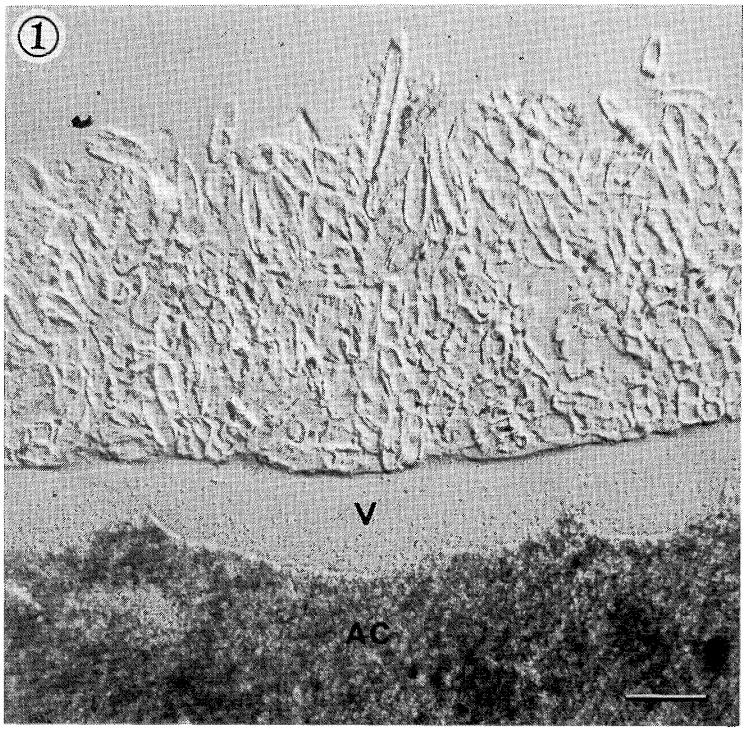


Fig. 1. TS through a biofilm composed chiefly of diatoms, growing on an antifouling varnish (V) over an anticorrosive coating (AC). Interference microscopy. Scale bar = 20μm.

2. Composition of algal biofilms

The composition of a biofilm depends primarily on qualitative and quantitative aspects of the inoculum. External factors such as the substrate, nutrient supply, competition and grazing all modify the colonization and growth processes.The sequence of events following immersion of a clean surface in water starts with adsorption of dissolved organic material including glycoproteins (Baier, 1980) and colonization by bacteria (Characklis & Cooksey, 1983; Marshall, 1985). Diatoms are the most common and abundant of the early algal colonizers and in certain situations the algal biofilm is almost entirely composed of diatoms with associated bacteria and a few entrapped miscellaneous unicells (Jackson & Jones, 1988). Diatoms are unicells characterized by the presence of an elaborately ornamented silica frustule and chloroplasts containing the pigment fucoxanthin which masks the chlorophyll, hence their golden brown colour. Each cell can range in size from a few to several hundred micrometres. Some forms are enclosed in mucilage sheaths (tubes) up to several centimetres in length, thereby assuming a filamentous appearance e.g. *Navicula ramosissima,* whilst others attach to the substrate by a variety of adhesive mechanisms including encapsulating mucilage e.g. *Amphora;* pads e.g. *Cocconeis*, and stalks e.g. *Achnanthes* (Daniel *et al.,* 1987). As with bacterial biofilms, only a few cells are needed to attach to a surface, as cell division rapidly gives rise to colonies which eventually coalesce to form a compact biofilm. Doubling times for populations of diatoms on glass slides immersed in the sea at Miami, Florida were between 11.7 and 27.4h depending on the time of year, similar to those found in the

laboratory cultures (Cooksey *et al.*, 1984). Diatom biofilms are typically up to 500μm in thickness (fig. 2) with some $2.7x10^5$ cells cm^{-2} (Hendey, 1951). Certain genera and species are of considerable importance in fouling of ships' hulls coated with antifouling paint (fig. 3). Species diversity appears to decrease with increasing effectiveness of the antifouling composition (Robinson *et al.*, 1985: Callow, 1986). *Amphora* spp. are most common on copper paints (see later) whilst diatom slimes dominated by species of *Achnanthes* and *Amphora* are commonly found on self polishing copolymer (SPC) paints containing both tributlytin and copper salts (fig. 3) (Callow & Edyvean, 1990).

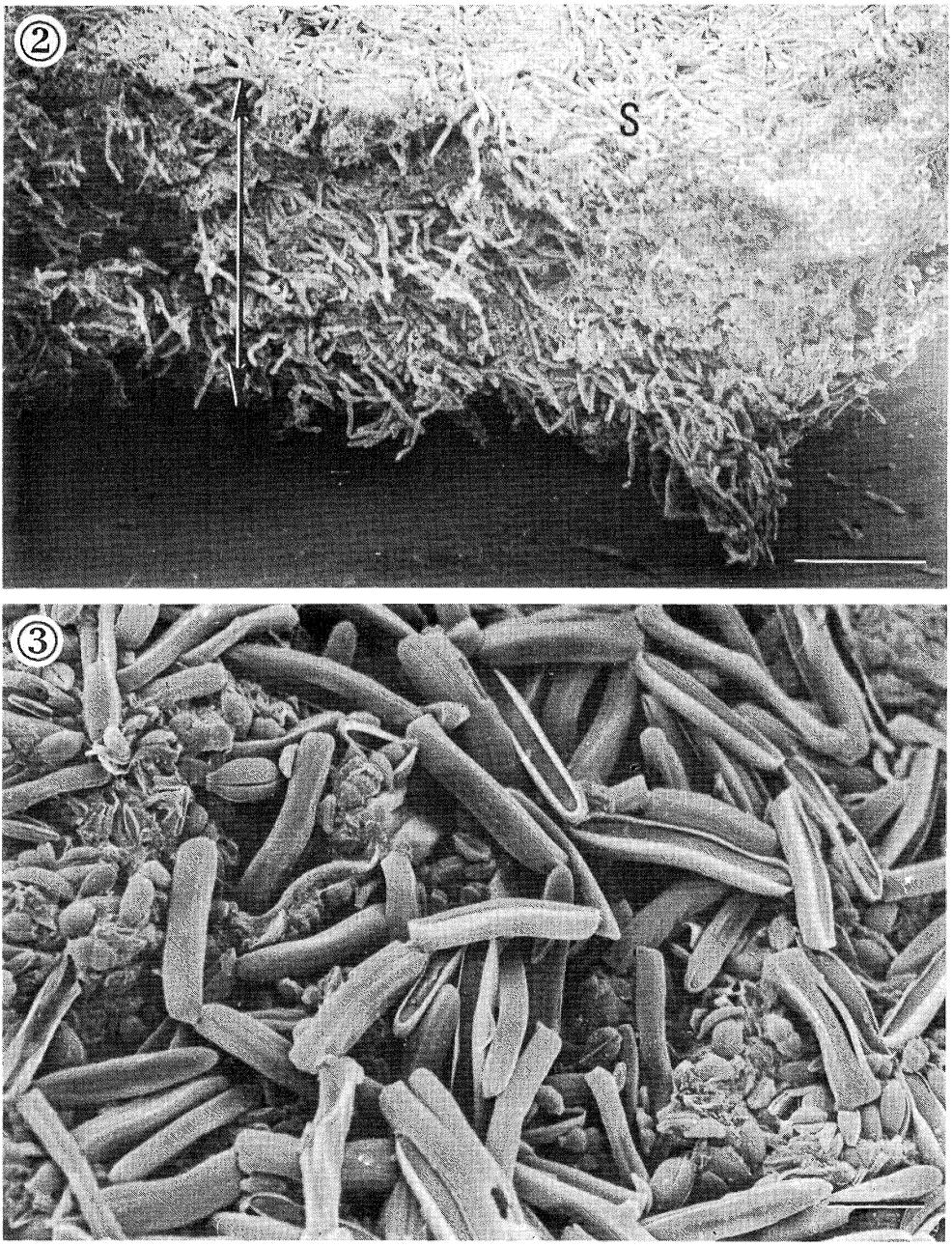
Many unicellular and filamentous green algae (Chlorophyceae) and cyanobacteria (blue-green algae) form colonies within algal biofims particularly in freshwater environments. In certain circumstances, especially in the aerial environment they may be the dominant members (Grant, 1982). For example, the green unicell *Pleurococcus* occurs in great profusion in all kinds of damp situations as a thin biofilm on stones, fences and tree trunks. *Trentepohlia,* a filamentous green alga, disfigures exterior stone and paintwork in humid tropical and subtropical regions regions by producing a green or orange covering (Wee & Lee, 1980). Cyanobacteria are simple prokarotic organisms without nuclei and with many other features in common with bacteria. Cyanobacteria are characterised by a blue-green colour, occasionally red, and exhibit a variety of growth forms from unicells to variously "branched" filaments. Cyanobacteria are commonly found as minor components of mixed algal biofilms but in certain circumstances they dominate e.g. on the vertical plates of heat exchangers in cooling towers (Lakatos, 1990) and in some industrial circulating cooling water systems (Ludyansky, 1991). The outstanding ability of cyanobacteria to withstand extreme conditions of temperature and drought as well as the ability of some to fix atmospheric nitrogen suits them well for survival in the aerial and nutrient depleted environments.

Macroalgal members of biofilms are chiefly green algae e.g. *Enteromorpha* and in the marine environment, small brown algae are also common e.g. *Ectocarpus.* All of these have a relatively simple growth habit which may consist of unbranched filaments e.g. *Ulothrix,* branched filaments e.g. *Ectocarpus, Cladophora* or a single layer of cells forming a tube e.g. *Enteromorpha.* Other relevant common features include the method of reproduction, through the production of large numbers of flagellate zoospores or gametes with the ability for rapid attachment to the substrate and adhesive rhizoids. In some species, germination gives rise to a thallus with a creeping, spreading habit allowing the algae to grow along the substrate and through an existing biofilm. Red algae, including encrusting forms, are occasionally found within biofilms in the marine environment particularly on static structures although they rarely dominate (see Fletcher *et al.*, 1984b; Callow & Edyvean, 1990). In view of their relatively minor role in biofilms red algae will not be considered in this review.

In addition to bacteria and the types of algae mentioned, the majority of biofilms incorporate casual algal members e.g. flagellates and desmids within the community along with the non-algal members e.g. protozoans, larvae, annelids and the non-biological components such as silt, sand and minerals. The biofilm should be regarded as a dynamic community in equilibrium with the environment and undergoing the same cycle of processes of growth, death, sloughing and regeneration associated with bacterial biofilms albeit in a less ordered and pronounced manner.

3. Settlement and adhesion of biofilm algae

Diatoms attach to the substratum by the production of mucilages which encapsulate the cells e.g. *Amphora,* or become elaborated morphologically to form pads e.g. *Cocconeis,* stalks e.g. *Achnanthes* or tubes e.g. *Navicula ramosissima* (Daniel *et al.*, 1987). The mucilage is synthesized



Figs 2 & 3. Scanning electron micrographs of a diatom biofilm growing on SPC antifouling paint. Fig. 2. Edge of biofilm (between arrows) and surface (S). Scale bar = 200μm. Fig. 3. Biofilm surface showing large cells of *Achnanthes* intermixed with cells of *Amphora*. Scale bar = 20μm.

in the Golgi apparatus, packaged into vesicles (Daniel *et al.*, 1987) and secreted at the plasmamembrane into the raphe slit (Webster *et al.*, 1985). The composition of diatom mucilages has similarities with bacterial exoploymers, being composed of acidic polysaccharides with smaller amounts of neutral polysaccharides (Jones *et al.*, 1982; Daniel *et al.*, 1987). In diatoms with encapsulating mucilage such as *Amphora,* the mucilage functions both as an adhesive and as a motility polymer allowing the diatoms to move by gliding over the substratum. The physiological requirements for adhesion in *Amphora* have been extensively studied by Cooksey and co-workers, reviewed recently in Wigglesworth-Cooksey & Cooksey (1992). The processes of synthesis and secretion of adhesive mucilage leading to motility and adhesion are prevented by uncouplers of energy metabolism, inhibitors of protein synthesis, cytoskeletal function and calcium transport. It is thought that a chemotactic sensing mechanism operates allowing the cell to sense a surface and then select a preferred niche in the substratum (Cooksey & Cooksey, 1988). The relative strength of attachment of diatoms increases with the length of time after settlement. Procumbent species like *Amphora,* appear to adhere more tenaciously to the substratum than stalked species and it is suggested that the former types are better able to withstand the shear stresses imposed by water movement (Woods & Fletcher, 1991). In common with adhesion of other types of cells and organisms, diatom adhesion is greater when cells attach to a roughened rather than a smooth surface. Cyanobacteria and non-motile green algae also attach by means of extracellular mucilages.

A completely different type of mechanism is encountered in the large majority of green and brown macroalgal members of the biofilm. Here, the attaching stage is a motile spore which swims by means of 2 or 4 hair-like flagella. Many algal spores have the ability to respond to environmental conditions which favour their subsequent survival. The best known responses are to surface topography (thigmotactic response), light (phototactic response) and the presence of chemicals (chemotactic response), reviewed by Fletcher & Callow (1992). The most well documented thigmotactic response is the preference of spores to settle and colonize roughened sufaces. Most zoospores or fused gametes are negatively phototactic, swimming towards areas of low light and thereby increasing their chances of settlement. The spores of some macroalgae appear to mimic those of some motile bacteria and diatoms, moving in response to nutrient gradients, thus allowing settlement in microhabitats more nutritionally favourable to growth.

The adhesives of all eukaryotic organisms such as diatoms and macroalgal spores are synthesised within the cell in the Golgi apparatus. However, the composition of the adhesive which attaches the spore to the substratum is more complex in macroalgal spores than that produced by diatoms. In the green alga *Enteromorpha,* the anterior region of swimming zoospores contains many Golgi-derived electron dense vesicles which contain glycoprotein adhesive. EM autoradiography indicates that the protein component is synthesised in the endoplasmic reticulum subtending the forming face of the Golgi bodies whilst the carbohydrate component is added later, probably within the vesicles after detachment from the Golgi bodies. On settlement, the vesicles rapidly discharge their contents, giving rise to a fibrillar adhesive which anchors the spore to the substratum. Initially the glue is susceptible to proteolytic attack but on "curing" (approx. 2h after settlement) the majority of spores cannot be dislodged and germination occurs. On the basis of EM studies, it appears that a similar mechanism of adhesive production and spore settlement operates in other motile spores (Jones *et al,* 1982; Fletcher & Callow, 1992). The tenacity with which cells, spores and germlings attach to the substratum is related both to the roughness and physico-chemical properties of the surface. As discussed previously, the strength of attachment is stronger to rough than to smooth surfaces (Woods & Fletcher, 1991). On low energy surfaces, the morphology of rhizoids of algal germlings is different to those which develop on high energy

surfaces (Fletcher *et al.*, 1984a). The strength of adhesion of algal biofilms is lower on low energy surfaces (Callow & Edyvean, 1990) and this phenomenon is being exploited in relation to fouling control (see below).

4. Physiology of algal biofilms.

In nature, algal biofilms contain mixed, structured communities of microorganisms including algae, bacteria, fungi, protozoa and, depending on the the aquatic environment, a mixed population of meiofauna. By comparison with natural open waters, where rates of photosynthetic and chemosynthetic primary production are low, submerged surfaces are relatively nutrient rich habitats particularly in oligotrophic, pelagic environments (Paerl, 1985). Surfaces provide an interface for the concentration of charged particles and molecules and may represent direct nutritional sources either due to their mineral or organic content. A range of inorganic molecules, including phosphate, iron, calcium, copper, other trace metals and a variety of organic molecules are readily concentrated on surfaces. Paerl and Merkel (1982) have shown cellular uptake rates for specific nutrients such as phosphate to be higher among particle-associated, as opposed to free-floating, bacteria. However, disagreement exists as to the trophic roles that surfaces play in some of the many habitats so far examined.

Within a biofilm, microenvironments or microzones (Paerl, 1985) develop in which particular physicochemical characteristics occur. Thus, concentrations of oxygen, CO_2, micronutrients and dissolved organic carbon (DOC) can vary spatially within a few micrometres and temporally in relation to diurnal, seasonal and annual changes in the surrounding environment. Various organisms within the biofilm become tightly coupled according to their nutritional requirements. Cycling of gases and nutrients, competition within and between species, predation and death all occur within the confines of a microscopic biofilm.

Algal colonies and filaments within biofilms are typically embedded in a matrix of exopolymers. Diatoms, cyanobacteria and bacteria secrete relatively large quantities of extracellular materials the composition of which varies according to the species present. They are characteristically complex carbohydrates including mucopolysaccharides and glycoproteins. Immediately overlying the surface of a submerged algal biofilm is a thin layer of static water known as the 'diffusive boundary layer', the depth of which depends on the surface texture of the film and the rate of movement of the overlying water. In static water the boundary layer may extend for 1mm above the film whereas at flow rates of 10cm s^{-1} the layer may be reduced to 100μm (Kuenen *et al.*, 1986). Dissolved gases and nutrients within the overlying water diffuse through the boundary layer immediately above the film and then through the exopolymer matrix before reaching cells of the biofilm. Conversely, waste gases and metabolic products are either recycled within the film or diffuse outwards to the overlying water. Some benthic algae, such as *Oedogonium kurzii*, display an 'inherent current demand' in that movement of water is essential for their survival (Whitford & Schumacher, 1961). The reason for this appears to be that currents greater than 15cm sec^{-1} produce a steeper diffusion gradient around the alga and as a result the uptake of nutrients and exchange of gases is increased. Measurements of biofilm activity, especially the exchange of gases such as oxygen and CO_2, have traditionally been achieved by placing a perspex chamber over the biofilm and then measuring the change in gas composition within the water above the film (Marker, 1976; Loeb, 1981). Such experiments can only give an approximation of fluxes within the film. Recently, microelectrodes have been developed for measuring pH, oxygen and sulphide which can be inserted into a biofilm to give a spatial resolution of 10-50μm (Revsbech *et al.*, 1983: Kuenen *et al.*, 1986; Glud *et al.*, 1992).

4.1. EFFECTS OF LIGHT ON ALGAL BIOFILMS

Algae utilise solar energy for photosynthesis with the end result that carbon is fixed to form complex organic compounds. Carbon is obtained as dissolved inorganic carbon (DIC) supplied by CO_2 in the atmosphere. Oxygen is generated as a by-product of photosynthesis. Thus whilst the biofilm is illuminated, CO_2 is utilised and oxygen produced but in darkness oxygen is utilised and CO_2 generated. Fig. 3a shows a typical example of steady state oxygen profiles in an algal biofilm collected from the surface of a trickling filter. Oxygen measurements were made in darkness and in light with an irradiance of 600μmol m^{-2} s^{-1} (Kuenen *et al.*, 1986). The oxygen content of the water 0.4mm above the biofilm is 240μmol O_2 l^{-1}. When the biofilm is illuminated, immediately above the surface of the algal film, within the diffusive boundary layer, the oxygen concentration increases linearly to 620μmol O_2 l^{-1}. Maximum oxygen concentration in the light is reached at about 0.2mm depth. Above this level algal cells are light saturated and below there is a slow decline in oxygen concentration resulting from light limitation which increases with increasing depth. In darkness the oxygen content of the water immediately above the algal film decreases linearly and reaches zero about 0.3mm below the surface of the film. As the process of oxygen evolution by photosynthesis is stoichiometrically related to the fixation of CO_2, the observed production of oxygen should be reflected in a change in bicarbonate concentration which can be followed indirectly by pH measurements as shown in Fig. 3b (Kuenen *et al.*, 1986). The curve illustrating the increase in pH, as a result of CO_2 withdrawal when the film is illuminated, is similar to the curve for the increase in oxygen production. When an illuminated biofilm is transferred from light to darkness there is a reduction in oxygen output and a related decline in pH. These results demonstrate that the oxygen concentration can change from zero to five times air saturation in less than 10min. Not all oxygen diffuses out of the biofilm. For instance, Kuenen *et al.*, (1986) calculated that about 60-70% of oxygen produced in algal films by photosynthesis was consumed within the films, at least one third of this may be due to photorespiration.

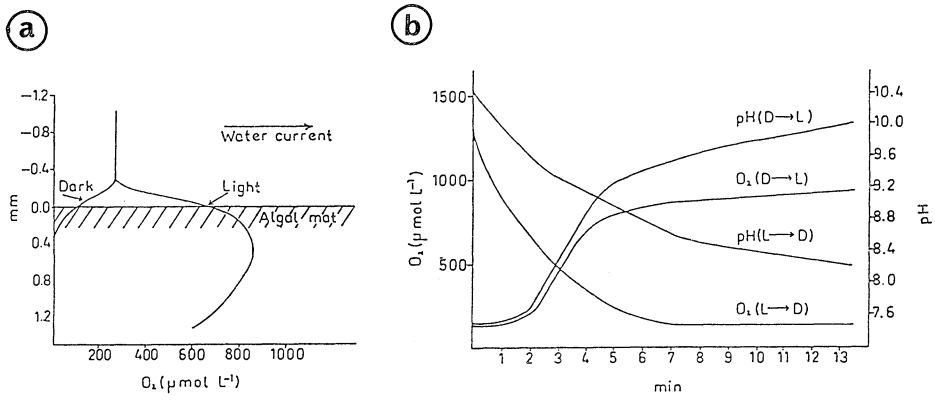


Fig. 4a. Typical profile of oxygen above and within an algal biofilm in the dark (left) and light (right). Zero depth is at algal film/water interface. Water flow rate 6cm s^{-1}, irradiance 600 μmol m^{-2} s^{-1}. Fig. 4b. Oxygen concentration and pH at a depth of 0.5mm in an algal biofilm as a function of time following transfer of biofilm from light to dark (L-D) and dark to light (D-L). (Illustrations redrawn after Kuenen *et al.*, 1986).

Photosynthesising biofilms create microenvironments characterised by high O_2/CO_2 ratios and substantial photorespiratory activity might therefore be expected. This is because ribulose 1.5 bisphosphate carboxylase catalyses two competing reactions (i) addition of CO_2 to ribulose 1.5 bisphosphate as in photosynthesis and (ii) oxygenation of ribulose 1.5 bisphosphate to produce glycollate and CO_2 as in photorespiration. At high O_2/CO_2 ratios increased oxygenase activity occurs resulting in the consumption of oxygen and generation of CO_2. Glud *et al.*, (1992) measured oxygen production by algal films flushed with water currents containing different concentrations of O_2 and HCO_3^-. They noted that in actively photosynthesising diatom films flushed with aerated water there was a decrease in respiration of about 17%, as measured by increase in oxygen production, when 5mM HCO_3^- was added to the overlying water. If the concentration of oxygen was increased, an increase in CO_2 was recorded equivalent to about 34% photorespiration. These experiments assume a constant rate of respiration throughout unaffected by changes in the O_2/CO_2 ratio. Biofilms containing *Oscillatoria* only exhibited slight increases in respiration when the O_2/CO_2 ratio was elevated. To overcome the potential for increased photorespiration it is possible that the intracellular DIC of *Oscillatoria* might be increased and thereby the CO_2 concentration, thus diminishing the oxygenase activity of the enzyme ribulose 1.5 bisphosphate carboxylase.

Productivity of algal mats varies greatly according to species composition, environmental conditions and supply of nutrients. For algal films in natural freshwater conditions, highest rates of primary production are normally encountered in Spring. Marker (1976) observed an inverse relationship between specific rates of gross photosynthesis and biomass with highest rates in May and June. After that time biomass increased to such an extent that photosynthesis was limited in thicker portions. Alternatively parts of the film sloughed off in flowing water or were grazed away. Similar results were observed in a small Alabama stream where, in April, the highest primary production recorded was 907mgC m^{-2} d^{-1} (Stock & Ward, 1991).

As algal films grow in thickness only the surface layers of the film remain photosynthetically active, lower layers are self-shaded and may become anaerobic. In films of *Oscillatoria submembranacea* up to 1mm thick, 98% of photosynthetic activity was associated with the surface of the mat whereas the bottom of the mat was brown in colour and anaerobic (Stock & Ward, 1991). The close proximity of actively photosynthetic cells and zones of anaerobiosis may result in the transfer of metabolic products that stimulate photosynthesis. Oxygen production by photosynthesis could also stimulate oxidative processes within the mat such as nitrification and methane oxidation.

4.2. NITROGEN FIXATION BY CYANOBACTERIA IN BIOFILMS

Cyanobacteria, in common with photosynthetic bacteria, can assimilate both CO_2 and elemental nitrogen and convert them into cell material (Fay, 1981). Nitrogen fixation is catalysed by the enzyme nitrogenase which can only function in the absence of oxygen. In many cyanobacteria nitrogen fixation is carried out in cells, called heterocysts, which are specifically modified to exclude oxygen. Nitrogen fixation by nitogenase to form ammonium ions is independent of light and many cyanobacteria can grow and fix nitrogen in darkness. However, light is required to produce the carbon skeletons required to combine with the ammonium ions to form glutamine. Nitrogen fixing cyanobacteria are capable of colonising habitats poor in nitrogen. Since they are capable of fixing both nitrogen and carbon from the atmosphere, their only other requirements are for a supply of phosphorus, trace elements, vitamins and other essential organic compounds.

Nitrogenase activity in cyanobacteria, like the rate of photosynthesis, increases linearly with increase in light intensity but may be depressed by the full intensity of solar radiation (Fay, 1981). Although heterocystous cyanobacteria do fix nitrogen in an oxygenic atmosphere, oxygen supersaturation can have a marked inhibitory effect. However, whereas increased oxygen in the region of 20-40% in the surrounding atmosphere inhibits photosynthesis leading to increased photorespiration, nitrogen fixation is only inhibited at oxygen concentrations in the range of 40% or more (Fay, 1981). Nitrogen fixation is inhibited in the presence of combined inorganic nitrogen including ammonium and nitrate. In nature, concentrations of ammonium (N) $3mgl^{-1}$ and nitrate (N) $9mgl^{-1}$ are enough to suppress nitrogenase activity. However, the utilisation of elemental nitrogen may be more important than anticipated since tuptake of combined nitrogen compounds from around an alga may create a microzone of nitrogen depletion. The diffusion coefficient of dissolved elemental nitrogen is higher than that of ammonium or nitrate ions (Fay, 1981).

4.3. RELEASE OF ORGANIC MATTER WITHIN BIOFILMS

The liberation of organic substances by actively photosynthetic cells is a common phenomenon well described for planktonic cells where 50% or more of fixed carbon may be released as DOC. In algal films the liberation of DOC has been studied in hot springs where *Synechococcus lividus*, growing at temperatures of 54-58°C, releases between 3-12% of total carbon fixed by photosynthesis (Bauld & Brock, 1974). Bacteria, fungi and heterotrophic algae, including some diatoms, in the algal film can then utilise these organic compounds as an energy source. In benthic mats, where algal and bacterial cells are closely packed, the transfer of organic material may be rapid and bacteria may serve as an effective sink for this material. The affinity of cyanobacteria for an extracellular supply of organic compounds is several orders of magnitude lower than the affinity of heterotrophic bacteria for the same compounds.
Within biofilms cycling of elements occurs in a manner analogous to the 'microbial loop' within nanoplankton systems (Azam *et al.*, 1983). Actively photosynthetic algae release DOC which provides a substrate for bacterial growth. Bacteria are grazed by protozoa and meiofauna which subsequently excrete ammonium and orthophosphate which can be reused by photoautotrophs. Likewise cycling of gases occurs with oxygen produced by photosynthesis being utilised by aerobic bacteria and CO_2 generated by respiration being used for photosynthesis.

4.4. ALGAL BIOFILMS AND CORROSION OF METALS

Although uniform biofilms of single algal species on metal surfaces may retard corrosion, most biofilms are not uniform in thickness or species composition and their heterogeneity leads to the formation of cathodic and anodic sites within the underlying metal (Edyvean & Videla, 1991). The cathodic oxygen reduction reaction becomes dominant at more highly oxygenated sites and as a result less oxygenated sites become anodic. Thus an illuminated biofilm consisting of colonies of cyanobacteria and bacteria will form cathodic and anodic sites respectively. Similarly, differences in thickness of the algal biofilm can cause the setting up of differential cells, with thinner portions of the biofilm allowing the passage of oxygen and hence becoming cathodic and the thicker portions becoming anodic. Corrosion is particularly pronounced in sea water where the concentrated salt solution acts as an electrolyte that completes the circuit between anode and cathode. The flow of electrons between the two electrodes produces a measurable current and localised corrosion in the form of crevices and pits occurs in the metal at the anodic site. Mixed algal/bacterial films can also alter the concentrations of ions beneath the biofilm thereby producing

chemical and pH concentration cells that act in a similar manner to oxygen concentration cells (Edyvean & Terry, 1983). Anaerobiosis at the base of biofilms may encourage the proliferation of sulphate reducing bacteria which promote corrosion (Edyvean & Callow, 1990; Edyvean & Videla, 1991).

4.5. BIOFILMS ON BIOCIDAL SURFACES

Unprotected surfaces soon become colonized by a community of macrofouling organisms including large brown algae (kelps) and hard (e.g. mussels, barnacles) and soft (e.g. hydrozoans) bodied animals. Antifouling paints are designed to prevent colonization by all types of fouling organisms. However, some organisms, especially diatoms (fig. 3) are highly resistant to biocides and consequently biofilms dominated by a few species of diatoms are commonly found on antifouling paint surfaces (see Callow & Edyvean, 1990). Copper and triorganotins are the most widely used biocides in antifouling paints and most of the research on biofilms growing on paint surfaces has been concerned with the effects of these two biocides. Soluble matrix paints, often referred to as conventional copper paints, contain copper and sometimes other biocides in a rosin matrix. Self polishing copolymer (SPC) paints are formed from a copolymer between tributlytin methacrylate and methyl methacrylate into which other biocides, primarily copper salts, are incorporated. On raft panels coated with conventional copper antifouling paint, Hendey (1951) recorded 97 species of diatoms from 29 genera, whilst Pyne *et al.*, (1986) recorded 91 species from 35 genera. In both studies the most abundant genus was *Amphora* (see fig. 3) also found to be the most common genus on both conventional copper and SPC paints (containing both tributlytin and copper) on 'in-service' ships (Daniel *et al.*, 1980; Callow, 1986). The number of diatom genera recorded on ships coated with SPC is lower than on raft panels presumably due to the higher leaching rates of biocides under dynamic as compared to static conditions. Diatom genera found on ships in addition to *Amphora* are *Amphiprora, Stauroneis, Navicula, Nitzchia, Navicula* and *Achnanthes* .

Amphora and *Amphiprora* are both highly resistant to copper (French & Evans, 1988). In *Amphora* resistance has been attributed to copper immobilization in membrane-bound intracellular bodies, thus keeping cytoplasmic levels low (Daniel & Chamberlain, 1981) and to binding of copper at the cell surface or to mucilage, the latter binding being removed by EDTA or cysteine (Robinson & Hall, 1990).

Triorganotins are lipid soluble compounds and thus rapidly enter cells where they function as energy transfer inhibitors in respiration and photosynthesis (Millner & Evans, 1980). Some macroalgae e.g. *Ectocarpus* and *Ulothrix* and diatoms e.g. *Achnanthes subsessilis* are resistant, possibly due to their ability for intracellular detoxification (Millner & Evans, 1981).

5. Problems caused by algal biofilms

The unsightly coloured appearance of exterior surfaces is the major disadvantage of algal biofilms on buildings. The consequences of algal biofilms in the aquatic environment are far more serious. On static submerged structures algae obscure the underlying substratum making inspection difficult and may alter the underlying microenvironment, thereby allowing proliferation of sulphate reducing bacteria and consequently promoting corrosion. (see Edyvean & Callow, 1990; Edyvean & Videla, 1991).

On ships, yachts and submarines, fouling of the hull and propellors causes increases in frictional resistance and drag as the vessel moves through the water, which result in speed loss, increased

noise and vibration. In order to maintain speed increased fuel consumption is necessary. Diatom slimes contribute significantly to speed loss. Lewthwaite *et al.* (1985) showed that a 1mm slime layer caused an 80% increase in skin friction together with a 15% loss of speed compared with values for the clean hull.

In fresh, hard water areas, algal biofilms become encrusted due to deposition of calcium carbonate. On boats these calcified biofilms are difficult to remove, often causing damage to the underlying substrate (see Heath *et al.*, this volume).

Algal biofilms growing within heat exchanger pipes impair function in the same manner as other types of fouling. In spray ponds and in water cooling towers, algae can disrupt water distribution, hinder cooling and block pipes and filters (Ludyansky, 1991). Algal biofilms in potable water storage water tanks or pipes are undesirable because the release extracellular DOC into the water and may impart tastes and odours (Hutson *et al.*, 1987).

6. Methods of control

Exclusion of light by the use of opaque materials may help is certain situations but in most instances this approach would be impractical. The most common method of reducing or eliminating algal biofilms is by the use of algicides or biocides which are either incorporated into coatings or dosed into water. In antifouling paints the most commonly used biocides which control both animal and algal fouling are copper salts and triorganotins where still permitted. In addition, a number of algicides, including diuron and triazine herbicides, are employed as well as in antialgal coatings for exterior use on buildings. With increasing health and safety requirements and increased environmental awareness, there is considerable interest in the development of non-toxic/non-polluting methods of biofilm control. One approach is to prevent the adhesion of cells by the use of non-stick or easy clean sufaces. Silicone elastomers (Callow *et al.*, 1986) and fluoropolymers (Griffiths, 1985) are easy clean low energy surfaces which so far have only found limited application as coatings on boats, static structures and pipelines. Adhesion may also be prevented by incorporation of substances which interfere with the chemotactic sensing of cells (Wigglesworth-Cooksey & Cooksey, 1992), or destroys the adhesive e.g. enzymes. Natural antifoulants, based on extracts from non-fouling marine invertebrates are being developed in the US although their activity is primarily against animals (e.g. Rittschof & Costlow, 1987). The use of algicides which degrade rapidly, either chemically or biologically are also being developed for incorporation into coating or dosing (e.g. Rossmoore, 1990). Effective reductions in the quantity of algicides used can be achieved through the use of synergistic combinations of compounds (Hunter & Evans, 1991) or through microencapsulation thereby allowing controlled release (Price *et al.*, 1991).

7. References

Azam, F., Fenchel, T., Field, J.G., Gray, J.S., Meyer-Reil, L.A. & Thingstad, F. (1983) The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser* 10, 257-263.

Baier, R.E. (1980) Substrata influences on the adhesion of microorganisms and their resultant new surface properties. In: Bitton, G, Marshall, K (eds) *Adsorption of microorganisms to surfaces.* Wiley, New York, pp. 59-104.

Bauld, J. & Brock, T.D. (1974) Algal excretion and bacterial assimilation in hot algal mats. *J. Phycol.* 10, 101-106.

Callow, M. E. (1986) Fouling algae from "in-service" ships. *Bot. mar.* 24, 351-357.

Callow, M.E. & Edyvean, R.G.J. (1990) Algal fouling and corrosion. In: Akatsuka, I (ed) *Introduction to Applied Phycology*. SPB Academic Publishing bv, The Hague, The Netherlands. pp. 367-387.

Callow, M.E., Pitchers R.A. & Milne, A. (1986) The control of fouling by non-biocidal systems. In L.V. Evans & K.D. Hoagland (eds), *Algal Biofouling*. Elsevier, Amsterdam, 145-158.

Characklis, W.G. & Cooksey, K.E. (1983) Biofilms and microbial fouling. *Adv. applied Microbiol.* 29, 93-138.

Cooksey. B., Cooksey, K.E., Miller, C.A., Rubin, R.W. & Webster, D. (1984) The attachment of microfouling diatoms. In: Costlow, J.D. & Tipper, R.C. (eds). *Marine Biodeterioration: An Interdisciplinary Study*. US Naval Institute, Annapolis, Maryland.

Cooksey, B. & Cooksey, K.E. (1988) Chemical signal-response in diatoms of the genus *Amphora*. *J. Cell Sci.* 91, 523-529.

Costlow, J. D. & Tipper, R.C. (eds) (1984) *Marine Biodeterioration: An Interdisciplinary Study*. US Naval Institute, Annapolis, Maryland.

Daniel, G.F. & Chamberlain, A.H.L. (1981) Copper immobilization in fouling diatoms. *Bot. Mar.* 14: 229-243.

Daniel, G.F., Chamberlain, A.H.L. & Jones, E.B.G.(1980) Ultrastructural observations on the marine fouling diatom *Amphora. Helgolander wiss. Meeresunters*. 34, 123-149.

Daniel, G.F., Chamberlain, A.H.L. & Jones, E.B.G. (1987) Cytochemical and electron microscopical observations on the adhesive materials of marine fouling diatoms. *Br. phycol. J.* 22, 101-118.

Edyvean, R.G.J. & Terry, L.A. (1983) Polarization studies of 50D steel in cultures of marine algae. *Int. Biodeterior. Bull.* 19, 1-11.

Edyvean, R.G.J. & Videla, H.A. (1991) Biological corrosion. *Interdisciplinary Science Reviews*, 16, 267-282.

Fay, P. (1981) Photosynthetic microorganisms. In: *Nitrogen fixation 1. Ecology*. Broughton, W.J. (ed.) Clarendon Press Oxford, 1-29.

Fletcher R.L. & Callow, M.E. (1992) The settlement, attachment and establishment of marine algal spores. *Br. phycol. J.* In press.

Fletcher, R.L. Baier, R.E. & Fornalik, M.S (1984a) The influence of surface energy on spore development in some common marine fouling algae. In *Proc. int. Congr. mar. Corros. Fouling*, 6, 5-8, August 1984, Athens, Greece. Marine Biology Volume: 129-144.

Fletcher, R.L., Jones, A.M. & Jones, E.B.G. (1984b) The attachment of fouling algae. In: Costlow, J.D. & Tipper, R.C. (eds), *Marine Biodeterioration: An Interdisciplinary Study*. Naval Institute Press, Annapolis, MD, 172-182.

French, M.S. & Evans, L.V. (1988) The effects of copper and zinc on growth of the fouling diatoms *Amphora* and *Amphiprora*. *Biofouling*, 1, 3-18.

Glud, R.N., Ramsing, N.B. & Revsbech, N.P. (1992) Photosynthesis and photosynthesis-coupled respiration in natural biofilms quantified with oxygen microsensors. *J.Phycol.*, 28, 51-60.

Grant, C (1982). Fouling of terrestrial substrates by algae and implications for control - a review. *Int. biodet. Bull.* 18, 57-65.

Griffith, J.R. (1985) The fouling release concept: A viable alternative antifouling coating? In: Smith, R. (ed.), *Polymers in a Marine Environment*. The Institute of Marine Engineers, London: 235-236.

Hendey, N.I. (1951) Littoral diatoms of Chichester Harbour with special reference to fouling. *J. r. micros. Soc.* 71, 1-86.

Hutson, R.A., Leadbeater, B. S. C. & Sedgwick, R.W. (1987) Algal interference with water treatment processes. *Progress in Phycological Research,* 5, 265-299.

Hunter, J.E. & Evans, L.V. (1991) Raft trial experiments on antifouling paints containing zineb and copper. *Biofouling*, 3, 113-137.

Jackson, S.M. & Jones, E.B.G. (1988) Fouling film development on antifouling paints with special reference to film thickness. *Int. Biodeterioration*, 24, 277-287.

Jones, A.M., Fletcher, R.L., Daniel, G.F. & Jones, E.B.G. (1982) Settlement and adhesion of algae. In: Mauchline, J. (ed.), *Fouling and Corrosion of metals in Seawater*. Scottish Marine Biological Association, Oban: 31-77.

Kuenen, J.G., Jorgensen, B.B. & Revsbech, N.P. (1986) Oxygen microprofiles of trickling filter biofilms. *Water Res.* 20: 1589-1598.

Lakatos, Gy. (1990) Study on the biofouling forming in industrial cooling water systems. In: Howsam, P (ed) *Microbiology in Civil Engineering*. FEMS Symposium 59. Chapman & Hall. pp. 81-94.

Lewthwaite, J.C., Molland, A.F. & Thomas, K.W. (1985) An investigation into the variation of ship skin resistance with fouling. *Trans. R.I.N.A.*, 127, 269-284.

Loeb, S.C. (1981) An *in situ* method for measuring the primary productivity and standing crop of the epilithic periphytic community in lentic systems. *Limnol. Oceanogr.* 26, 394-399.

Ludyansky, M. (1991) Algal fouling in cooling water systems. *Biofouling,* 3, 13-21.

Marker, A.F.H. (1976) The benthic algae of some streams in Southern England. *J. Ecol.* 64, 359-373.

Marshall, K. (1985) Mechanisms of bacterial adhesion at solid-water interfaces. In: Savage, D.C. & Fletcher, M (eds), *Bacterial Adhesion,* Plenum Press, New York. pp. 133-161.

Millner, P.A. & Evans, L.V. (1980) The effects of triphenyltin chloride on respiration and photosynthesis in the green algae *Enteromorpha intestinalis* and *Ulothrix pseudoflacca. Pl. Cell Envir.* 3: 339-348.

Millner, P.A. & Evans, L.V. (1981) Uptake of triphenyltin chloride by *Enteromorpha intestinalis* and *Ulothrix pseudoflacca. Pl. Cell Envir.* 4: 383-389.

Paerl, H.W. (1985) Influence of attachment on microbial metabolism and growth in aquatic ecosystems. In: Savage, D.C. & Fletcher, M. (eds.) *Bacterial adhesion,* Plenum Press, New York. 363-400.

Paerl, H.W. & Merkel, S. (1982) The effects of particles on phosphorus assimilation in attached vs. free floating microorganisms. *Arch. Hydrobiol.* 93, 125-134.

Price, R., Patchan, M., Clare, A., Bonaventura, J. & Rittschof, D. (1991) Elimination of rosin from vinyl antifouling coatings by the use of novel cylindrical channel-forming microtubules. In: *Polymers in a Marine Environment.* The Institute of Marine Engineers, London.

Pyne, S., Fletcher, R.L. & Jones, E.B.G. (1986) Diatom communities on non-toxic substrata and two conventional antifouling surfaces immersed in Langstone Harbour, South coast of England. In: Evans, L.V. & Hoagland, K.D. (eds) *Algal Biofouling*. Elsevier, Amsterdam. pp. 101-113.

Revsbech, N.P., Jorgensen, B.B., Blackburn, T.H. & Cohen, Y. (1983) Microelectrode studies of the photosynthesis and O_2, H_2S and pH profiles in a microbial mat. *Limnol. Oceanogr.* 28, 1062-1074.

Rittschof, D. & Costlow, J.D. (1987) Macrofouling and its management by non-toxic means. *Advances in Aquatic Biology and Fisheries.* 1-11.

Robinson, M.G. & Hall, B.D. (1990) Reversal of copper toxicity in *Amphora coffeaeformis*: role of externally bound copper. *Biofouling*, 2, 179-189.

Robinson, M. G., Hall, B.D. & Voltolina, D. (1985) Slime films on antifouling paints: short-term indicators of long-term effectiveness. *J. Coatings Technology*. 57, 35-41.

Rossmoore, H.W. (1990) The interaction of formaldehyde, isothiazolone and copper. *Int. Biodeterioration,* 26, 225-235,

Stock, M.S. & Ward, A.K. (1991) Blue-green algal mats in small streams.*J. Phycol.,* 27, 692-698.

Webster, D.R., Cooksey, K.E. & Rubin, R.W. (1985) An investigation of the involvement of cytoskeletal structures and secretion in gliding motility of the marine diatom *Amphora coffeaeaformis*. *Cell Motility,* 5, 103-122.

Wee, Y.C. & Lee, K.B. (1980) Proliferation of algae on surfaces of buildings in Singapore. *Int. biodet. Bull.* 16, 113-117.

Whitford, L.A. & Schumacher, G.J. (1961) Effect of current on mineral uptake and respiration by a freshwater alga. *Limnol. Oceanogr.* 6, 423-425.

Wigglesworth-Cooksey, B. & Cooksey, K.E. (1992) Can diatoms sense surfaces?: state of our knowledge. *Biofouling*, 5, 227-238.

Woods, D.C. & Fletcher, R.L. (1991) Studies on the strength of adhesion of some common fouling diatoms. *Biofouling*, 3, 287-303.

BACTERIAL AND ALGAL INTERACTIONS IN BIOFILMS

K.E. COOKSEY
Montana State University
Bozeman, MT 59717
U.S.A.

1. Introduction

Biofilms that are found on illuminated surfaces usually contain phototrophs. These surfaces can be rocks in mountain streams (Haack & McFeters, 1982a, b), water-treatment equipment (Ludyansky, 1991) or various natural and man-made structures in the marine environment (Paul, et al, 1977; Callow, 1986). There has been a great deal of work on the interactions between bacteria and phototrophs (usually phytoplankton) in the ocean water column (Wiebe & Smith, 1977; Ducklow, et al, 1986), but far less where the organisms are sessile. The reasons for this imbalance are not difficult to define. Whereas it is comparatively easy to work with organisms in suspension, it is far more difficult to study attached organisms. Even the comparatively simple procedure of sampling organisms growing on a surface is difficult (Wimpenny, et al, 1989). It follows that any analyses after sampling will be that much more difficult and prone to artefact. The interest in mixtures of suspended cells derives from our continuing goal of understanding global cycling of elements. Environments are not monospecific and all species play some role in the cycling process. At the moment, these specific roles are hard to define. Currently this area of science has assumed a far greater importance since the onset of fears concerning global warming trends.

There is no doubt that it is the proximity of cells to one another that most dictates the degree to which they can interact (see Haack & McFeters, 1982a for a sample of micrographs). In a planktonic situation, cells are several micrometers apart (depending of course on the cell concentration and the size of the cells). In a biofilm the cells are in contact or separated only by a matrix polymer (Cooksey, 1992).

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 163–173.

2. Types of Interaction

The interaction can be syntrophic or mutualistic. In illuminated films containing bacteria and algae, this means that bacteria can be influenced by algal metabolism, the reverse situation or there will be a mutual influence. In general the interaction is likely to be chemically mediated. Even tactic interactions are transduced chemically (Van Houten, 1990). The molecules involved can be large or small. Table 1 shows some of the molecules that could influence cell-cell interactions.

TABLE 1. Bases for cellular interaction in biofilms.

Compound	Potential involvement in consortial metabolism
O_2	produced by phototrophs, electron acceptor for heterotrophs.
CO_2	produced by heterotrophs, carbon source for phototrophs.
Organic N or C	N may be limiting to both phototroph or heterotroph. Carbon could be used by either component of the biofilm.
Sources of P or S	No information on excretion, but lysis could make these available.
Bioactive molecules, allelochemicals, antibiotics	Compounds such as cyclic AMP or chemoattractants may influence biofilm establishment. Vitamins, especially B_{12}(algae).
Extracellular polymeric substances	Diffusion limitation. Causes steep chemical gradients. Limits cellular motility.
DNA	Not likely to be transferred from procaryote to eukaryote or vice versa, but procaryote to procaryote transfer likely.

Oxygen is produced by the photoautotrophic member of the consortium which in the case of an algal/bacterial film is the alga. Because of potentially reduced diffusion in a film there is a possibility that this compound can increase in dissolved concentration over that normally found in water, i.e. about 0.25mM. The initial enzyme in the pathway responsible for CO_2 fixation is capable of reacting with O_2, as well as CO_2. In fact the name used for this enzyme is ribulose biophosphate carboxylase-oxygenase (Rubisco). When reaction with O_2 occurs, the photosynthetic ultimate product is glycolate, i.e. no CO_2 is fixed. Glycolate is metabolized by some algae (Codd, et al, 1969; Cooksey, 1970; Lord & Merrett, 1971) through the agency of a glycolate dehydrogenase. Formation and/or activity of this enzyme seems to be related to the endogenous oxygen tension (Tolbert, 1980), but other explanations have been put forward (Cooksey, 1970). Oxygen is required for aerobic respiration in heterotrophs. If we postulate that the heterotrophic component of the consortium is bacterial, then oxygen will be removed as an electron acceptor in heterotrophy. Furthermore, the glycolate produced by the action of Rubisco acting in its oxygenase mode will provide a respiratory substrate-glycolate. This scheme is shown in Fig. 1.

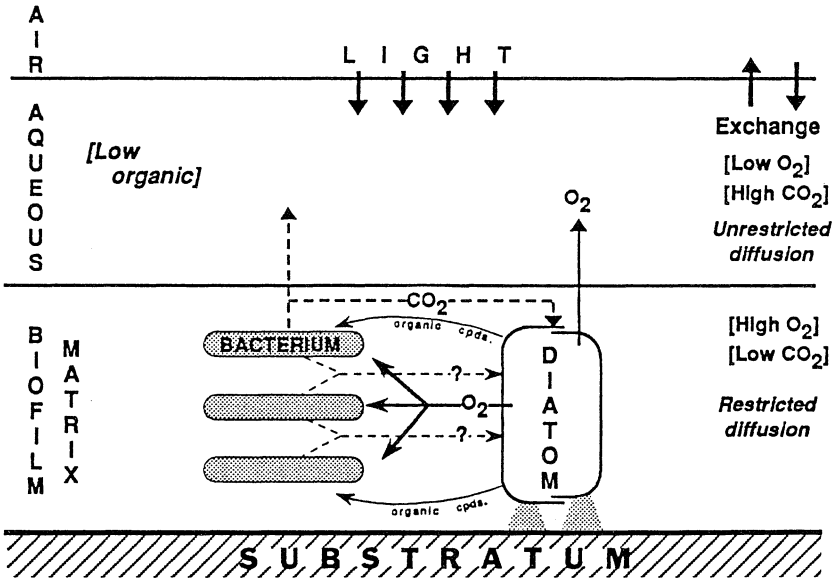


Figure 1. Possible metabolic interactions within a biofilm containing a phototroph and a heterotroph. For details, see text.

There is a large literature (Wiebe & Smith, 1977; Cole, 1982) on the excretion of organic materials by planktonic algae. Very few of the compounds have been identified (except for glycolate) but it is reasonable to assume that any compound of which there is a sufficiently large intracellular pool will also be found external to the cell - albeit in low concentrations. Glutamic acid is such a molecule. This could supply a source of nitrogen as well as carbon and energy to heterotrophic bacteria in the film. The same is probably true for phosphorus and sulfur compounds, but I cannot quote examples of these.

It is important to bear in mind that a biofilm is a very large concentration of cells. In any case in nature where this happens there is also always an enrichment of viral particles. For many years, the viral content of the marine environment has been ignored. However, recently the viruses have been found in the planktonic environment in very large numbers (c.10^{10} L^{-1}) (Berg et al, 1989; Torella & Morita, 1979; Suttle et al, 1990). There is absolutely no reason why the sessile environment should be different. If lytic viruses specific for algae or bacteria are present, all cell contents of one organism become available to the other. It is quite likely that in some cases the sloughing phenomenon, commonly seen in mature natural biofilms, involves viral infection. I am aware of no studies on this aspect of the phenomenon, however. It has been pointed out that the bare patches seen in natural biofilms can be the result of protozoan grazing (Jackson & Jones, 1991).

Many of the molecules released from one cell will not have specific effects on another. i.e. they will merely be sources of carbon, nitrogen or other structural components of the cell. Other molecules, however, may have specific signalling functions. For example, extracellular cyclic adenosine monophosphate causes aggregation in slime molds (Segall, 1990). Other molecules may cause specific cellular events by binding to membrane receptors. Although it is likely that chemotaxis is not important in the initial formation of a biofilm, it may well be important in its subsequent reorganization. Bacteria are certainly likely to be attracted to algal products. Some of the diatoms found in biofilms are tactic to sugars and to at least one amino acid (glutamate) (Cooksey & Cooksey, 1988).

None of the compounds mentioned so far are macromolecular, yet these too are either released or synthesized extracellularly by components of the film. For instance, extracellular polymeric substances (EPS), the global name for the extracellular slime found in films, reduces molecular diffusion and cell motility (see Cooksey, this volume). DNA is known to exchange between organisms that are in very close proximity (transformation). Although this is known in nature for bacterial/bacterial exchanges, no instances are known for bacterial/algal or algal/algal exchanges.

3. Evidence for Interactions

As mentioned earlier, much of the information on the interactions of cells in biofilms has been inferred from results obtained on homogenized films. For instance, Haack & McFeters (1982a, b) separated components of a homogenized biofilm that had

been harvested from rocks in an oligotrophic alpine stream. They tried to minimize artefacts resulting from the leaking of cells by treating the film with formaldehyde before processing it. However, they did not demonstrate that their rather vigorous homogenization procedures did not cause cells to leak. Nevertheless, their results showed that the catabolic activity of the sessile bacteria was tied to the metabolic state of the phototrophic organisms (mostly diatoms in this case). Bacterial heterotrophic activity, but not cell number, increased as the phototrophic population declined.

A gentler approach was taken in our laboratory. Diatom cells (*Amphora coffeaeformis*) grow attached to laboratory glassware if the concentration of calcium of the medium is higher than 2mM (seawater is approximately 10mM). So strong is their adhesion to glass, that the overlying medium can be decanted without disturbing the cells. When cells were grown in the presence of $NaH^{14}CO_3$, the medium which had been sterile-filtered contained acid stable radioactivity that could be assimilated by a marine bacterium, *Vibrio proteolytica* (Cooksey, unpublished data). Although this procedure was very gentle in terms of causing cellular damage it did not utilize a mixed film and thus again the interaction had to be inferred. One of the first papers to report quantitative information on the in situ metabolism of a mixed film of partially controlled composition concerned a biofilm of *Amphora sp.* and unspecified bacteria (Jensen & Revsbech, 1989). The information was gathered by the use of an oxygen microelectrode. The construction and use of these very small probes (10μm tip) has been reviewed (Revsbech & Jorgensen, 1986). The mixed species biofilm was grown in a bioreactor of unusual design. Diatoms (unialgal, but not axenic) were inoculated into one side (A) of a double chamber which was separated from the other side by a 0.2 micrometer (pore size) polycarbonate filter. The medium in the chamber containing the diatoms was phosphate-free. The alternate chamber contained similar medium, plus phosphate. The only source of phosphate in chamber A therefore was by diffusion from chamber B through the polycarbonate filter. It was on the polycarbonate filter that the diatom biofilm formed. A film of 1-1.5mm could be produced which was ideal for the in situ measurement of photosynthesis and respiration. Jensen & Revsbech were able to demonstrate that photorespiration took place in the diatom (Figures 2a,b). Indeed it seems that a biofilm that experiences high light will also be bicarbonate limited - ideal conditions for photorespiration. Photorespiration results in the formation and presumed excretion of glycolate. Further, the fact that oxygen production was increased when the bacterial content of the diatom culture used to produce the film was reduced by the use of antibiotics, supports the notion of bacterial use of photosynthetically produced O_2. This paper stresses algal metabolism however, rather than the interactions. Two points which were somewhat incidental to the thrust of the paper are very important here. Oxygen electrodes can be used to measure oxygen diffusion coefficients. This paper demonstrates what has been assumed in many other communications - O_2 diffusion in the film is just about half that in free medium i.e. 1.1 cm^2 sec^{-1} in the film compared to 2.1 cm^2 sec^{-1} in medium. A second observation mentions the difficulty

in preparing thick (1mm) films of *Amphora* when an attempt had been made to reduce the bacterial content of the film. Gas bubbles (presumably photoproduced O_2) caused the film to slough. These workers suggest that where a coherent adhesive film is produced, it is due to the production of extracellular polymers by the bacterial component. I suggest that although this is certainly possible, the extracellular polymer could be produced by the diatoms in response to the presence of the bacteria. Woods & Fletcher, (1991) made parallel observations when examining the ability of axenic populations of *Amphora coffeaeformis, var. perpusilla* to withstand removal from a surface subjected to hydraulic shear stress in a Fowler cell (Fowler & McKay, 1979; Dudderidge et al, 1982). They noted that sloughing took place in the cell (axenic culture) but this was not observed on ship bottoms where the film presumably contained bacteria. Nevertheless, Woods & Fletcher, in contrast to Jensen & Revsbech, considered that it was the inclusion of other organisms in the film that caused its sloughing.

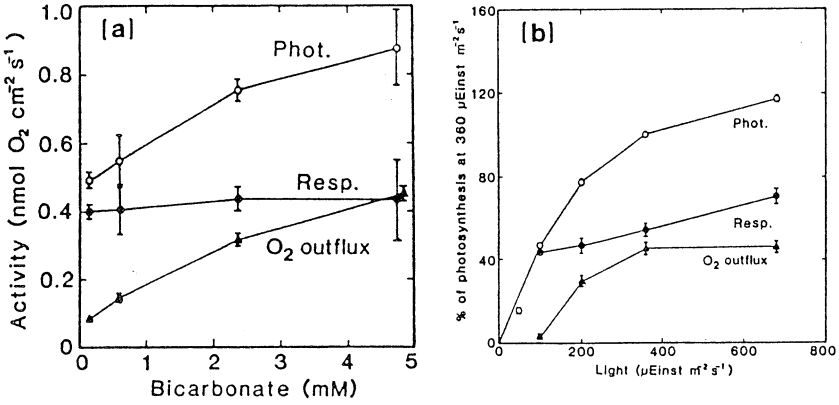


Figure 2. Gross photosynthesis, O_2 out of flux and respiration in a mixed diatom-bacterial film. (a) Measurements made at 360μEinsteins $m^{-2}sec^{-1}$ as a function of initial bicarbonate concentration. (b) Measurements made as a function of light intensity. (Reprinted with permission from Jensen & Revbech, FEMS Microbiology Ecology, Elsevier.)

Paerl & Gallucci, (1985) give an example in which the association of a heterotrophic bacterium (pseudomonad) and a phototroph (*Anabaena oscillariodes*) is specific. The association which enhanced N_2-fixation in the cyanobacterium, involved the chemotaxis of the pseudomonad to compounds secreted by phototroph. A role for the pseudomonad is in reducing local O_2 concentrations and thus maintaining optimal conditions for N_2-fixation. No such specific association has been shown for attached algal-bacterial communities. Reasons for this may be the concentration of research effort on mature natural communities and monospecific films formed in the laboratory.

An interesting and practical interaction in mixed films has been noticed by Little, et al, (1991). These workers found that the corrosion potential, E_{corr} of stainless steel which was covered with a marine diatom and bacterial film was light dependent. Although the illumination level used in the experiments (20-50 ft. candles) was no where near that saturating for photosynthesis for the average diatom species, (c. 500-1000 ft. candles in the scale used by the authors) significant ennoblement of E_{corr} was found (+200mV). The E_{corr} could be regarded as the sum of all electrochemical reactions taking place at the metal-biofilm interface and as such could serve as an indicator of cell-cell interactions within the film. This work has been extended by Dowling, et al, (1992). Phototrophic metabolism of a cyanobacterium, *Anabaena* on the surface of stainless steel electrodes produced perceptible oscillations in E_{corr} which followed the light/dark cycle used in the incubation. The presence of a heterotrophic bacterium in the films was without effect on the E_{corr}. The authors suggest that this is because little organic carbon was available to the heterotroph. In terms of the current paper we can say that the phototroph (*Anabaena*) and the heterotroph (*Delaya marina*) did not interact under these conditions. The system described below would be an ideal one with which to study the influence of illumination on E_{corr} in mixed films where interactions have already been documented. Murray, et al, (1986) formed algal-bacterial consortia on polystyrene surfaces from axenic cultures of the marine diatom *Amphora coffeaeformis* and the marine bacterium *Vibrio proteolyticus*. Organisms were attached to the surfaces at cell densities of $5x10^4$ diatoms and $5x10^6$ bacteria cm^{-2}. Although the cell ratio of 1/100 was chosen for this study, there is no reason other ratios could not be achieved. We found that the algal/bacterial consortia showed higher rates of incorporation of [^{3}H] thymidine into the film than did biofilms composed solely of bacteria. When the film was 16 hrs. old, the differential was fourfold. After 70 hrs., it had risen to 16 fold. Note that it is the age of the film that is given here, not the time for the incubation with labelled thymidine (1 hr) [Figure 3]. When the incubations were carried out in the dark, little or no thymidine was incorporated, i.e. DNA synthesis in the heterotrophic bacterium was light-dependent. Since the measurement of radioactivity in the harvested fixed film measured the total incorporation into the cold trichloroacetic acid insoluble fraction of the entire film, we decided to seek further assurance that tritium was incorporated only into the bacterial component. The results in Figure 3 had already suggested this however, i.e. incorporation in diatoms was not significantly different from that in formalin-killed controls. ($P>0.05$). Mixed cell films were attached to plastic cover slips and incubated for 20h in the light (c. 100μ Einsteins m^{-2} s^{-1}) when [^{3}H] thymidine was added. After 1 h further, the cells were fixed in formalin (5%), stained with Hoechst 33258 (Paul, 1982) and radioautographed by the method of Brock & Brock, (1968). Photomicrographs of the films under phase, epifluorescent and mixed phase and epifluorescent illumination respectively were made. Under phase illumination the black silver grains could be seen clearly to be associated with the pale blue stained bacteria. The algal nuclei showed as bright blue areas of the algal cells. Developed silver grains were not seen

in association with algal nuclei in either monoalgal or mixed biofilms. We concluded from this and the results in Figure 3 that *A. coffeaeformis* did not incorporate [^{3}H] thymidine, whereas *V. proteolyticus* did - but only significantly in films where it was in the presence of the diatom.

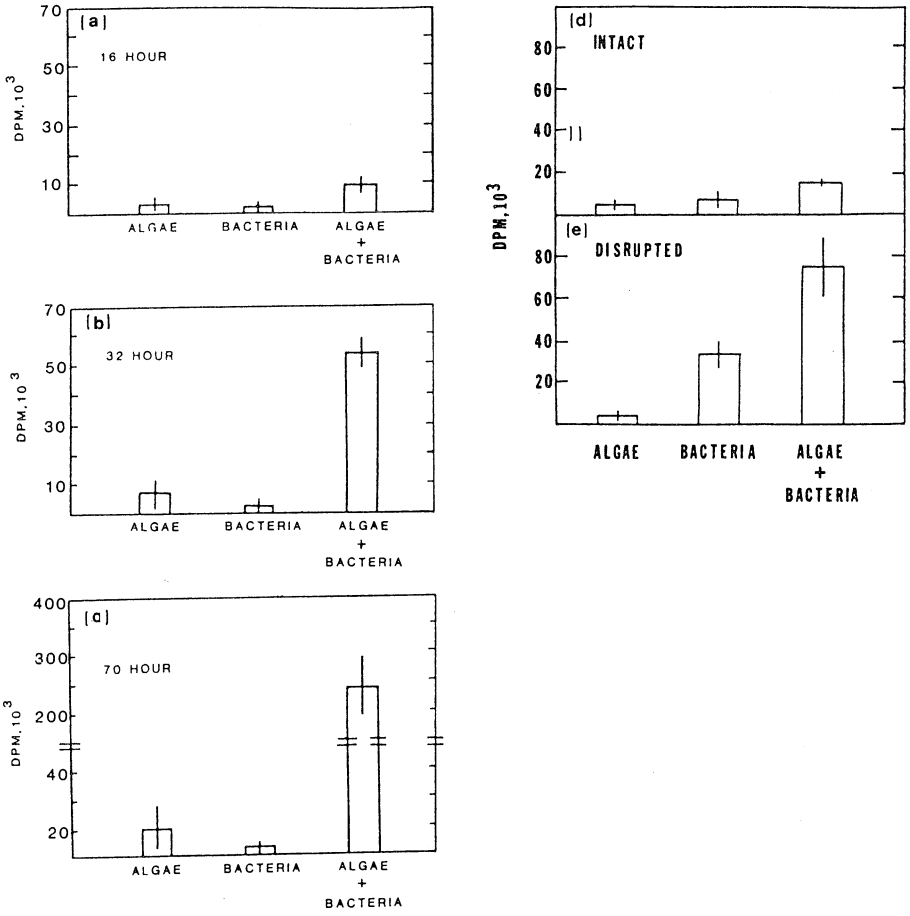


Figure 3. Incorporation of [^{3}H] thymidine into intact monobacterial, monoalgal and mixed algal-bacterial films. (a), (b), (c) incorporation with time (h.). (d) intact films incubated for 48h. before measuring the rate of [^{3}H] thymidine incorporation. (e) similar to (d) but the film was harvested using a sterile rubber policeman before addition of thymidine. Note incorporation into algae in panel (a) is not significantly different from the formalin-killed control. (Reprinted with permission from Murray-Cooksey & Priscu, Applied & Environmental Microbiology, American Society for Microbiology).

I believe that this system is open for further development. It should be possible to introduce another organism so making a tripartite film. It should also be possible to measure the interaction as a function of biocide challenges and also the extent of the interaction as a function of metabolizable substrate in the overlying water. Does the interaction cease if external heterotrophic growth substrates are available? Is the interaction changed if the nitrogen source in the medium is altered? The questions are many.

Murray, et al did not follow this route but did make one further observation of importance here (Murray, et al, 1987). Rates of [^{3}H] thymidine incorporation into the cold TCA-insoluble material by bacteria cultured without an exogenous carbon source were 4.3 times greater when activity was measured after disruption of a monobacterial biofilm [Figure 3d,e]. When diatoms were present in the film, the disrupted rate was five times greater than the in situ rate. These differences are not likely to be caused by problems of diffusion of the [^{3}H]-thymidine because the films were cellular monolayers. Further we do not believe the difference in rates is due to the fact that in the disrupted film all the cell area is available for uptake of the substrate, whereas we calculate only about 80% is available when the bacteria are attached to a surface (about 20% is in contact with the substratum). The difference in rates is several hundred percent, far greater than the 20% possible by a consideration of the relative cell membrane area available for uptake of the radiolabelled material. Thus we contend that measurements made on the physiology of harvested and disrupted films do not reflect that of the intact films. Others have made similar observations with naturally occurring films (Ladd, et al, 1979).

4. Conclusions

The study of cell-cell interactions in biofilms is difficult. Two approaches stand out as potential solutions to the difficulty. These are the use of microelectrodes for the measurement of in situ chemistry, and the production of mixed films of known and controlled composition. Harvesting of films and homogenization before measurements are made is not an acceptable approach, except to make biomass estimates.

5. Acknowledgement

We thank the U.S. Office of Naval Research for the support of work in our laboratory and Karen Bess for the preparation of the manuscript.

6. References

Berg, O., Borsheim, K.Y., Bratbak, G., and Heldal, M. (1989) "High abundance of viruses found in aquatic environments". Nature (Lond.) 340, 467-468.

Brock, T.D., & Brock, M.L. (1968) "The application of micro-autoradiographic techniques to ecological studies". Mitt. Int. Ver. Theor. Angew. Limnol. 15, 1-29.

Callow, M.E. (1986) "Fouling algae from "in service" ships". Botanica Marina 29, 351-257.

Cole, J.J. (1982) "Interactions between bacteria and algae in aquatic ecosystems". Annu. Rev. Ecol. Syst. 13, 291-314.

Codd, G.A., Lord, J.M., and Merrett, M.J. (1969) "The glycolate oxidising enzyme of algae". FEBS Letter 5, 341-342.

Cooksey, K.E. (1970) "Glycolate 2:6 dichlorophenol-indophenol oxidoreductase in *Chlamydomonas reinhardtii*". Plant Physiol. 48, 267-269.

Cooksey, K.E. (1992) "Extracellular polymers in biofilms" in Melo et al (eds.), Biofilms: Science and Technology, Kluwer Academic Publishers, Dordrecht, The Netherlands, this volume.

Cooksey, B., and Cooksey, K.E. (1988) "Chemosensing in diatoms of the genus Amphora". J. Cell Sci. 91, 523-529.

Dowling, N.J.E., Guezennec, J., Bullen, J., Little, B., and White, D.C. "Effect of photosynthetic biofilms on the open-circuit potential of stainless steel". (1992) Biofouling: Submitted for publication.

Ducklow, H.W., Purdie, D.A., Williams, P.J.L., and Davies, T.M. (1986) "Bacterioplankton, a sink for carbon in a coastal marine plankton community". Science 232, 865-867.

Dudderidge, J.E., Kent, C.A., and Laws, J.F. (1982) "Effect of surface shear stress on the attachment of *Pseudomonas fluorescens* to stainless steel under defined flow conditions". Biotech. Bioeng. 24, 153-164.

Fowler, A.W., & McCay, A.T. (1979) "The measurement of microbial adhesion", in R.C.W. Berkeley, J.M. Lynch, J. Melling, and B. Vincent (eds.) Microbial Adhesion to Surfaces, Ellis Horwood Ltd., Chichester, pp. 143-161.

Haack, T.K., and McFeters, G.A. (1982a) "Microbial dynamics of an epidithic mat community in a high alpine stream". Appl. Environ. Microbiol. 43, 702-707.

Haack, T.K., and McFeters, G.A. (1982b) "Nutritional relationships among microorganisms in an epilithic biofilm community". Microbiol. Ecol. 8, 115-126.

Jackson, S.M. and Jones, E.B.G. (1991) "Interactions within biofilms: The disruption of biofilm structure by protozoa". Kiel. Meeresforsch. 8, 264-268.

Jensen, & Revsbech, N.P. (1989) "Photosynthesis and respiration of a diatom biofilm cultured in a new gradient growth chamber". FEMS Microbial Ecol. 62, 29-38.

Ladd, T.I., Costerton, J.W., and Geesey, G.G. (1979) "Determination of heterotrophic activity in epilithic microbial populations", in J.W. Costerton & R.R. Colwell (eds.), Native Aquatic Bacteria: Enumeration, Activity and Ecology. Am. Soc. Test. Mat., Philadelphia, pp. 180-185.

Little, B., Ray, R., Wagner, P., Lewandowski, Z., Lee, W.C., Characklis, W.G., and Mansfeld, F. (1991) "Impact of biofouling on the electrochemical behaviour of 304 stainless steel in natural seawater". Biofouling 3, 45-59.

Lord, J.M. and Merrett, M.J. (1971) "The growth of *Chlorella pyrenoidosa* on glycollate". J. Exp. Bot. 22, 60-69.

Ludyansky, M.L. (1991) "Algal fouling in cooling water systems". Biofouling 3, 13-21.

Murray, R.E., Cooksey, K.E., and Priscu, J.C. (1986) "Stimulation of bacterial DNA synthesis by algal exudates in attached algal-bacterial consertia". Appl. Environ. Microbiol. 52, 1177-1182.

Murray, R.E., Cooksey, K.E., and Priscu, J.C. (1987) "Influence of physical disruption on growth of attached bacteria". Appl. Environ. Microbiol. 53, 2997-2999.

Paerl, H.W., and Gallucci, K.K. (1985) "Role of chemotaxis in establishing a specific nitrogen-fixing cyanobacterial-bacterial association". Science 227, 647-649.

Paul, J.H. (1982) "Use of Hoechst dyes 33258 and 33342 for enumeration of attached and planktonic bacteria". Appl. Environ. Microbiol. 43, 939-944.

Paul, R.W., Kuhn, D., Plafkin, J.L., Cairns, J., and Croxdale, J.G. (1977) "Evaluation of natural and artificial substrate colonization by scanning electron microscopy". Trans. Amer. Micros. Soc. 96, 506-519.

Revsbech, N.P. and Jorgensen, B.B. (1986) "Microelectrodes: Their use in microbial ecology". Adv. Microbial Ecol. 9, 293-352.

Segall, J.E. (1990) "Mutational studies of amoeboid chemotaxis using *Dictyostelium discoideum*", in J.P. Armitage & J.M. Lackie (eds.), Biology of the Chemotactic Response. Cambridge University Press, Cambridge, pp. 241-272.

Suttle, C.A., Chan, A.M., and Cottrell, M.T. (1990) "Infection of phytoplankton by viruses and reduction of primary productivity". Nature 347, 467-469.

Tolbert, N.E. (1980) "Photorespiration," in D.D. Davies, (ed.), The Biochemistry of Plants Vol. 2. Metabolism and Respiration, Academic Press, New York, pp. 488-523.

Torella, F., and Morita R.Y. (1979) "Evidence for high incidence of bacteriophage particles in waters of Yaquina Bay, Oregon: ecological and taxonomic implications". Appl. Environ. Microbiol. 37, 774-778.

Van Houten, J. (1990) "Chemosensory transduction in Paramecium", in J.P. Armitage and J.M. Lackie (eds.), Biology of the Chemotactic Response, Cambridge University Press, Cambridge, pp. 297-321.

Wiebe, W.J., and Smith, D.F. (1977) "Direct measurement of dissolved organic carbon release by phytoplankton and incorporation by microheterotrophs". Marine Biol. (Berlin) 42, 213-223.

Wimpenny, J.W.T., Peters, A., and Scourfield, M. (1989) "Modeling spatial gradients", in W.G. Characklis & P.A. Wilderer, (eds.), Structure and Function of Biofilms, Wiley Ltd., Chichester, pp. 111-127.

Woods, D.C., and Fletcher, R.L. (1991) "Studies on the strength of adhesion of some marine fouling diatoms". Biofouling 3, 287-303.

FUNGAL BIOFILMS ON METALLIC SURFACES

M.MOTA and L. MELO
Dep. Eng. Biológica
Universidade do Minho
Largo do Paço 4719 Braga Codex
PORTUGAL

1. Introduction

There are not many publications devoted to the study of the attachment of fungi to surfaces, the available articles being in general limited in scope as well as in perspective.

Fungi have not drawn much attention as far as adhesion is concerned. Yet , in the vast majority of interactions between fungi and the environment, prior attachment is needed.

This is the case of the biodeterioration of wood, as well as of other well known examples such as the moulding of stored foodstuffs, decay of paint and paint films, leather, books, paintings, adhesives and even glass (the etching of glass lenses in the tropics is of fungi's responsibility) (Onions et al, 1986).

The adhesion of filamentous fungi to plastics and plasticizers was extensively studied by Pankhurst et al. (1972), Olson et al. (1977) and Pantke (1977) under the point of view of biodeteroration. The same perspective was adopted in the work of Mogilnitskii et al (1987), who investigated the biodeterioration of PVC. A study of the adhesion to perspex by marine fungi was also performed by Hyde et al (1989).

Growing attention is being recently paid to the detrimental effects of mycotoxins liberated by fungi into foodstuffs - namely ochratoxins and aflatoxins - since the latter have been incriminated as potent carcinogens and there are proofs of the toxicity of ochratoxins for mammals(Linsell, 1977; Ames, 1989).

The ochratoxins are of interest in food processing for at least three reasons: (1) they are toxic to several animal species, and probably to man also, (2) some are highly resistant to heat and (3) many fungi are capable of producing ochratoxins at storing temperatures, i.e., below 10ºC (Frazier and Westhoff, 1988).

Therefore, the prevention of the attachment of conidia to food processing surfaces is important, since spores are one of the major propagation ways of filamentous fungi. Only two references, both published by the same research team, were found in the literature (Kaznacheev et al, 1986, 1989). These two papers were devoted to the attachment onto polymers, namely cellophane and epoxy resins. As cellophane and epoxy resins are

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 175–180.

widely used materials for food packaging, this may be considered a valuable work in the field of food processing.

Nevertheless, adhesion to important surfaces in food processing, such as metals and metal alloys, have unfortunately been little considered so far. A survey in the literature revealed one publication concerning the corrosion of metals by biofilms of fungi (Rosales et al, 1990) dealing with the corrosion produced by *Cladosporium resinae*. *Cladosporium* strains are not major food contaminants (Jay, 1978), let alone the strain used in this work, in which food contamination was not a matter of concern.

The other work studying adhesion to metals was that of Andreyuk et al (1982). In this case, the biofouling was studied on steel surfaces dipped in seawater, corresponding to conditions which in no way can be compared to those found in food spoilage and contamination (except for the special case of curing).

In summary, we can see that the adhesion of food spoilage fungi such as *Aspergillus, Penicillium, Candida*, etc., to food processing surfaces has been poorly studied so far. To give another example of the lack of research in this field it is just enough to point out that there was not a single communication on biofilm formation by fungi in the important filamentous fungi symposium that was recently held in Yugoslavia (Physiological Aspects of Product Formation by Filamentous Fungi - Programme and Abstracts - 4-7 Nov 1990, Gozd Martuljek).

The study of fungi adhesion activity is also important in the field of wastewater treatment. As a matter of fact, fungi play an important role in the treatment of liquid wastes, being either beneficial, by removing xenobiotics (Glaser,1990) or detrimental, when they give rise to filamentous bulking (Atlas and Bartha, 1987). Furthermore, main treatment processes either aerobic - viz. trickling filters and rotating biological contactors (RBC) - or anaerobic - anaerobic filters and expanded beds, for example - are attached-growth waste treatment processes (Metcalf and Eddy, 1991).

Little published work can be useful to this field. Indeed, the above mentioned publications concern either plastic supports which are not used in attached-growth processes, or the wrong fungi. Once more, this is an open field to active investigation.

In the present work, an experimental set-up is described and used for the assessment of the adhesion ability of filamentous fungi to different metallic surfaces. The experimental conditions used in this preliminary work are equivalent to those normally found both in food processing and in waste treatment.

2. Materials and Methods

ORGANISMS

The following fungi were used:
Aspergillus niger van Tieghen was purchased from the FCL collection (Lisbon, Portugal) ; *Geotrichum candidum* Link ex Persoon and *Candida lipolytica*(Harrison) Diddens & Lodder were kindly furnished by Dr. Amaral-Collaço from LNETI (Lisbon, Portugal).

CULTURE MEDIUM

The medium was composed of 0.5% peptone and 0.3% yeast extract dissolved in tap water.

EXPERIMENTAL SET UP

The experimental set-up is depicted in figure 1.The metal plates were turned to the central axis of the flask. The rotation speed of the magnetic bar was adjusted to the desired value with the help of a stroboscopic meter.

METALS AND ALLOYS

The metals and alloys assayed were stainless steel , aluminium, copper, zinc and brass. The assayed metallic plates were polished, thoroughly rinsed, dried and weighed before the attachment runs. Plastified wires were used to suspend the plates and dip them at the same level in the culture medium.

PREPARATION, INCUBATION AND MEASUREMENTS

The 500 mL erlenmeyer flasks containing 200 mL of culture medium and the metallic plates were sterilized in an autoclave at 121°C for 15 minutes. The inocula were obtained by transferring 2 loops of a slant culture to a 100 mL conical flask containing 25 mL of culture medium with the above described composition. After an incubation in an orbital shaker for 48 hours at 30°C, 10 mL were pipetted into the 500 mL flask containing the metal plates. The cultures were allowed to grow for 1 week at 30°C as represented in fig.1. The stirring speed was adjusted to 150 rpm. After 1 week growth, the metal plates were carefully removed from the culture, dried at 105°C till constant weight and weighed again in a 0.1 mg precision balance. The culture medium was filtered through previously weighed 0.45 micron membrane filters, which, after a drying period at 105°C, were weighed again to measure the biomass dry weight suspended in the culture.

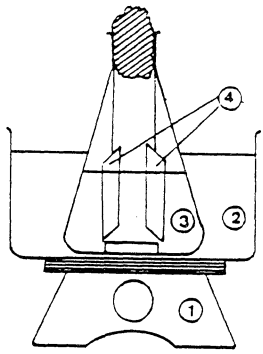


Figure 1 - Experimental set-up for the assessment of biofilm formation:
1 - magnetic stirrer, 2 - water bath, 3 - erlenmeyer flask, 4 - metal plates.

3. Results and Discussion

Each dry weight allowed to assess 1) the mass of filamentous fungi attached per unit mass of the metal plate and 2) the fraction of the whole biomass which became attached to the plates.

The results obtained for each strain and metal are represented in Tables 1 and 2.

TABLE 1 . Results obtained (in grams), for the total grown biomass, the suspended biomass and the adhered biomass, corresponding to each strain and each kind of metallic plate.

Metal/alloy	Biomass	*G.candidum*	*C.lipolytica*	*A. niger*
	Plates	0.0247	0.0123	1.6872
STEEL	Suspended	0.3130	0.0610	3.7650
	Total	0.3372	0.0733	5.4522
	Plates	0.0281	0.0386	0.4796
ALUM.	Suspended	0.2104	0.3973	3.7081
	Total	0.2385	0.4359	4.1877
	Plates	0.0149	0.0250	0.0309
BRASS	Suspended	0.1841	0.0385	0.7854
	Total	0.1990	0.0635	0.8163
	Plates	0.7012	0.2595	0.5178
ZINC	Suspended	0.3581	0.1757	0.2575
	Total	1.0593	0.4352	0.7753
	Plates	0.0256	-	0.0025
COPPER	Suspended	0.0812	-	0.0529
	Total	0.1068	0.0018	0.0554

TABLE 2 . Proportion (in %) of biomass that adhered to the metallic plates for each strain

	Steel	Al	Brass	Zinc	Copper
G.candidum	7.3	11.8	7.5	66.2	24.0
C.lipolytica	8.2	8.9	39.4	59.6	-
A.niger	30.9	11.5	3.8	66.8	4.5

From these results, it may be seen that zinc promotes a significant growth of all the strains. Furthermore, in the case of zinc, the growth is mainly located on the plates for all the strains. Curiously, zinc has been shown to inhibit the adhesion of bacteria (Duddridge et al, 1981; Vieira et al.,1992) The influence of the other metals is largely strain-dependent which may be seen when the second best metal for adhesion is identified. The second best metal after zinc is copper for *G.candidum* , brass for *C.lipolytica* and steel for *A.niger* . The comparison of the results obtained for zinc, copper and brass leads to the conclusion that copper is the main responsible for the lower growth in the brass plates, because the growth in the presence of zinc alone is several times greater, except for *A.niger*.

Liberation of metallic ions to the culture medium was also evaluated. Analysis by atomic absorption spectrophotometry showed that i) concentrations of copper and zinc in the spent culture medium were 10 times higher than in the fresh medium, ii) the concentration of iron decreased during the experiment, meaning that there was a consumption of that metal, and iii) aluminium concentration did not vary. The significant increase in the concentrations of copper and zinc in solution may justify different physiological behaviours of the strains as far as total growth is concerned.

4. Conclusions

As the metal plates were turned to the rotation axis, the adhering cells were undergoing shear forces all along the growing and attachment process. As a consequence of the conical geometry of the flask and of the proximity of the walls, there are different liquid speeds at different levels on the metallic plates. Thus, the linear liquid speed cannot be calculated exactly. Nevertheless, although it is not a procedure as sophisticated and rigourous as those described by Fletcher (1990), it allowed us to assess the adhesion of various fungi to different surfaces. Furthermore, it proved 1) that if we want to build a food processing facility, the materials to be used must be chosen according to fungi more susceptible to contaminate the plant and 2) that each organism has a different adhesion ability, varying according to the metal in use, which means that adhesion studies should be performed in each particular case. This procedure is now in course of application to other fungi and materials.

AKNOWLEDGEMENTS

The authors want to aknowledge the contribution in laboratory work the students A.P.Castro and N.Coelho have given to this research.

5. References

Ames, BN (1989) 'Chemicals, cancers, causalities and cautions', Chemtech, Oct., 590-598.

Andreyuk, EI, Kopteva, ZhP, Yanova, SB, et al. (1982) 'Growth of microorganisms on the surfaces of steels 09G2 and 10KhSND in seawater and different seasons of the year', Mikrobiol.Zh., Vol.44, 3, pp.16-19 (in russian).

Atlas,RM and Bartha,R (1987) Microbial Ecology: Fundamentals and Applications, 2nd Ed., Benjamin Cummings Pub Co.,MenloPark, Cal.

Duddridge,JE, Kent,CA and Law,JF (1981) 'Bacterial adhesion to metallic surfaces' in Progress in Prevention of Fouling, Nottingham University, 137, April.

Fletcher,M (1990) 'Methods for Studying Adhesion and Attachment to Surfaces', in R.Grigorova and JR Norris (eds.), 'Methods in Microbiology', vol.22, Academic Press, London, pp. 251-283.

Frazier,Wc and Westhoff,DC (1988) Food Microbiology, 4th Ed., McGraw-Hill Book Co., New York.

Glaser,JA (1990) 'Hazardous waste degradation by wood degrading fungi', in D.Kamely, A.Chakrabarty and G.S.Omenn (eds.),'Biotechnology and Biodegradation', Gulf Pub. Co., Houston, pp.267-284.

Hyde,KD, Moss,ST and Gareth Jones,EB (1989) 'Attachment studies in marine fungi', Biofouling, Vol.I, pp. 287-298.

Jay.J (1978) Modern Food Microbiology, D Van Nostrand Co., New York.

Kaznacheev,IV, Gumargalieva,KZ and Moiseev,Yu.V(1986) ' Adhesion of *Aspergillus niger*conidia to different polymeric surfaces', Dokl.Akad.Nauk.USSR, Vol.291, 5, pp.1241-1244 (in russian).

Kaznacheev,IV, Gumargalieva,KZ, Moiseev,Yu.V and Mironova,SN (1989) 'Change in the level of adhesion of conidia in relation to the composition of the cell wall and the age of the culture', Mikrobiol.Zh., Vol.51., 6, pp. 63-67 (in russian).

Linsell, CA (1977)'Aflatoxins', in JJ Lenihan and WW Fletcher (eds.) Environment and Man, Vol.6. The Chemical Environment, Blackie, Glasgow and London, pp. 121-136.

Metcalf and Eddy (1991) Wastewater Engineering: treatment, disposal, reuse, McGraw-Hill Book Co., 3rd Ed.,New York.

Mogilnitskii,GM, Sagatelyan,RT, Kutischeva,TN, Zhukova,SV, Kerimov,SI and Parfenova,TB (1987) 'Deterioration of the protective properties of PVC coating by microorganisms', Zasch, Met., Vol.23, 1, pp. 173-175 (in russian).

Onions, AHS, Allsopp and Eggins, HOW (1986) Smith's Introduction to Industrial Mycology, 7th Ed., Edward Arnold, London.

Osmon,JL and KlausMeier,RE (1977)'Techniques for assessing biodeterioration of plastics and plasticizers', in AH Walters (ed.), Biodeterioration Investigation Techniques, Applied Science, London, pp. 77-94.

Pankhurst,ES, Davies,MJ and Blake,HM (1972) 'The ability of polymers or materials containing materials to provide a source of carbon for selected microorganisms', in AH Walters and EH Hueck van der Plas (eds.), Biodeterioration of Materials, Applied Science, London, pp. 76-90.

Pantke,M (1977) 'Test methods for evaluation of susceptibility of platicized PVC and its components to microbial attack', in AH Walters (ed.), Biodeterioration Investigation Techniques, Applied Science, London, pp. 51-76.

Rosales,BM and Esteso,MA (1990) ' Microbiologically induced corrosion of metals by biofilms of fungi', Rev.Iberoam.Corros.Prot., Vol.21, 5, pp. 171-175 (in spanish).

Vieira,MJ, Oliveira,R, Melo,L , Pinheiro,MM and Martins,V (1992) 'Adhesion of biofilms formed by *Pseudomonas fluorescens*', Colloids and Surfaces (accepted).

EFFECTS OF BIOFILM FORMATION ON PLASMID SEGREGATIONAL STABILITY AND EXPRESSION

STEVEN W. PERETTI
Department of Chemical Engineering
North Carolina State University
Raleigh, North Carolina
27650-7905 USA

JAMES D. BRYERS
The Center for Biological Engineering
Duke University
Durham, North Carolina
27706 USA

1. Introduction

Recombinant plasmids are a significant tool for biotechnological research and have enormous commercial potential for the expression of foreign genes in procaryotic and eucaryotic organisms. Two major impediments to widespread utilization of genetically-engineered expression systems within natural ecosystems are: *(1)* the instability of the plasmid under a variety of conditions, and *(2)* the lack of knowledge regarding the fate of recombinant DNA in natural ecosystems. Governmental regulations have thus far severely restricted the development of processes involving the exposure to the environment of geneticallymanipulated organisms. Not surprisingly, significant effort has been spent in determining the mechanisms involved in plasmid loss from cells, and in initial studies of the fate of genetically-engineered DNA sequences in the environment.

A plasmid is deemed unstable if it either undergoes rearrangement *(structural)* or is not absolutely inherited by progeny *(segregational)*. Such phenomenon can have significant effects on the outcome of cell cultivation processes. It has been well established, both experimentally and theoretically, that plasmid maintenance and cloned gene expression reduce the overall growth rate of the plasmid-bearing cell relative to the plasmid free cell [Seo and Bailey,1985; Peretti, Bailey and Lee,1987; Zund and Lebek,1980; Lauffenburger,1987]. Reduction of copy number [Grandi, *et al.*, 1981] and loss of plasmids from populations under continuous culture have been reported in many cases [Wood and Peretti, 1990; Kadam, *et al.*, 1987; Nakazawa, 1978; Meacock and Cohen, 1980], even in the presence of selective pressure [Peretti and Bailey (1987); Kadam, *et al.*, 1987]. Plasmid loss has been shown to occur in *E. coli* [Seo and Bailey, 1985; Uhlin, *et al.*, 1979; Fredrickson, 1977; Klemperer, *et al.*, 1979; Engberg and Nordstrom, 1975; Jones, *et al.*, 1980] as well as in *Bacillus* [Wood and Peretti, 1990; Kadam, *et al.*, 1987; Nakazawa, 1978] and Pseudomonas [Meacock and Cohen, 1980]. The discovery of a *par* locus [Skogman, 1983] has lead to many efforts to genetically stabilize plasmid segregation. While this has not always met with great success [Gerges, 1988], recent work with the *parB* locus has been extremely promising [Kadam, *et al.*, 1987; Meacock and Cohen, 1980; Gerges, 1988; Bailey and Ollis, 1986].

That immobilization might stabilize a plasmid-bearing population in suspension can be shown mathematically [Dykhuizen and Hart, 1983]. Plasmid persistence in suspended cultures has been observed in cases of where the plasmid-bearing cell was at a growth rate disadvantage [Adams, *et al.*, 1979; Grandi, *et al.*, 1981; Inloes, *et al.*, 1983] and has been directly attributed to biofilm formation and cell sloughing from the film [Inloes, *et al.*, 1983]. Inloes *et al.*, (1983) reported the maintenance of a plasmid-containing strain of *E. coli* in the absence of selection pressure when immobilized in a hollow fiber membrane.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 181–198.

The effect of immobilization on plasmid stability has been investigated by de Taxis du Poët *et al.* (1986). Plasmid-bearing *E. coli* were immobilized in κ-carrageenan beads that were subsequently fluidized in a chemostat operated at a volumetric residence time of 15 minutes. Cells extracted from the beads and resuspended were shown to have the same plasmid-loss frequency as suspension-cultured cells, yet it was reported that immobilization enhanced the stability of the plasmid-bearing population. Nasri *et al.* extended this analysis to three genetically-different *E. coli* hosts (HB101,W3101, and B) using the same plasmid (pTG201), and again reported that, without antibiotics, the fraction of cells carrying plasmid in the beads was greater that one would find in suspension cultures. In free suspension, all planktonic cells exhibit varying degrees of plasmid instability; when immobilized all three strains exhibited stable plasmid maintenance for the duration of the culture. When plasmid-bearing and -free cells were coimmobilized, plasmid free cells did not overrun the culture. Their results suggest that increased plasmid stability was not due to either plasmid transfer between cells or to increases in copy number. Sayadi *et al.* repeated Nasri's work with just *E. coli* B (pTG201) but under different growth nutrient limitations. This third study found a decreasing specific growth rate, increased plasmid copy number and cloned gene activity but decreased stability. They also found that immobilization, in the absence of antibiotic selection, increased stability of the plasmid under glucose, nitrogen, or phosphate limitations but not for magnesium limited growth. Unfortunately, all the above reports are difficult to interpret. The gel-immobilized cultures are grown in a chemostat operated at a different volumetric residence time, much higher than possible for the suspension cultures used for comparison. None of the three studies determined the intrinsic growth rates of the cells inside the gel bead. Thus it is difficult to ascertain whether the apparent increase in stability is due to decreased growth rate in the bead resulting from nutrient mass transport limitations, plasmid transmission between cells or other mechanisms.

Many studies of plasmid transfer rates for suspended cells indicates that this transfer can occur at significant rates under a variety of conditions and organisms [Freter, *et al.*, 1983; Morrison, *et al.*, 1978; Stewart and Carlson, 1986], including *Pseudomonas* grown in the presence of competing organisms from a natural ecosystem [Morrison, *et al.*, 1978]. Studies of plasmid transfer in aquatic systems show that transduction [Stewart, *et al.*, 1983], transformation [Stewart and Carlson, 1986; Gealt, *et al.*, 1985], and triparental mating [Clewel, 1981] are all possible means of plasmid movement in these systems.

Studies of *Streptococcus faecalis* [Clewel, *et al.*, 1982; Navarro and Durand, 1977] further indicated that conjugative plasmids that transfer at a relatively low frequency in suspension (10^{-6} per donor), when immobilized to a surface, exhibit significantly higher transfer frequencies ($>10^{-4}$ per donor).

There are a number of strategies to improve plasmid stability in recombinant bacterial systems (Table 1). It comes as no surprise that immobilization might be responsible for alterations in cellular behavior. Many different immobilized cell systems have exhibited altered metabolism. Studies done with immobilized *Saccharomyces* have indicated alterations in productivity [Tyagi and Ghose, 1982; Doran and Bailey, 1986], macromolecular composition [Doran and Bailey, 1987], and regulation of glycolytic oscillations [Hattori, 1972]. *E. coli* has been found to exhibit changes in optimal growth conditions and product yields [Ou and Alexander, 1974], while *Bacillus* has an altered cell morphology [Graham and Istock, 1978]. In studies of plasmid transfer in sterile soil, Graham and Istock, (1978; 1979) found that transformation occurred in *B. subtilis* in the absence of plasmids or transducing bacteriophage. Similarly, transduction [Zeph, *et al.*, 1988; Weinberg and Stosky, 1972; and conjugation [Weinberg and Stosky, 1972; Monbouquette and Ollis, 1988] have been observed for soil microorganisms in natural biofilms. Kumar and Schügerl (1990) provide an excellent review of the observed increases in plasmid

retention, cloned gene expression, and system protein productivity seen in a variety of immobilized cell (bacterial, yeast, and animal cell) systems. Unfortunately, no concise explanation exists for these observed improvements in plasmid stability.

This brief section indicates the ubiquitous occurrence of plasmid movement when considering the fate of genetically-engineered DNA in the natural environment. These transfer processes occur in both gram-positive and - negative organisms, in suspended and biofilm-bound communities. Quantification of the risks involved in release of plasmid-bearing cells to the environment, be it inadvertent or intentional, will therefore require quantitative, mechanistic information regarding both the survival and mobility of the original host/plasmid system in these environments (suspended and immobilized) and the frequency of plasmid transfer to indigenous microorganisms from the original host.

Table 1. Protocols for enhancing plasmid stability.

System	- antibiotic resistance markers on plasmid plus antibiotics in media - amino acid growth requirements - temperature shifts - two-stage bioreactor operation, delayed induction - immobilization
Genetic Approaches	- auxotrophic mutants (amino acids) - suicidal genes (*cer, par, ζ par B* locus) - regulation of gene expression - recombinant deficient host (rec A), internal selection pressure (gamma lysogens, streptomycin dependency)

2. Factors Affecting Plasmid Stability and Expression in Suspended Cultures

Three primary factors affect the segregational stability of a plasmid in a bacterial population and the expression of the genes therein cloned. These are *(1)* the number of plasmids per cell at the time of cell division, *(2)* the presence of different partition loci, and *(3)* the strength of the promoter for the cloned gene.

A statistical analysis of plasmid loss frequency and plasmid copy number indicates that the rates of plasmid loss exhibited for many host/vector pairs are what would be expected if plasmid segregation between progeny were completely random. A cell containing 50 plasmids could give rise to a daughter cell containing anywhere from 0 to 50 plasmids. Plasmid copy number control is obviously, then, very important. The initiation of plasmid replication is controlled at the level of transcription, and as such is highly dependent on the strength of the promoter that directs the transcription of the replication primer and on the presence of a *rop* protein. The plasmid copy number is not, however, a constant for any given plasmid, but depends as well on the growth rate of the cell. Peretti and Bailey (1987) showed theoretically that slower growing cells have a greater proportion of their RNA polymerase available for non-chromosomal transcription, including the initiation of plasmid replication. These cells can thereby maintain a higher number of plasmids than those growing more rapidly. This phenomenon has been widely reported from experimental studies as well [Weber and San, 1987; Wouters, *et al.*, 1980].

Meacock and Cohen (1980) first reported a partition locus and described the increase in segregational stability it conferred upon the plasmids in a suspended population. Since that time several more such loci have been identifled and characterized. Only two of these loci have been found to be directly related to the physical partitioning of the plasmids (attachment to membranes) and these have been associated with stringently maintained, low copy number plasmids such as F' in E. *coli.* The others can be more correctly described as killing loci. A good example of this class is the *parB* locus. This system produces mRNA *(hok)* for a protein that destroys the transmembrane potential of the cell and for an anti-sense RNA *(sok)* to this message. When the plasmid is present in the cell, the relative rates of transcription and degradation of these two RNA species is balanced such that no protein is produced. Once the plasmid is no longer present in the cell, the *sok* RNA is rapidly degraded, exposing the ribosome binding site of the *hok* mRNA. Subsequently, the protein is produced and the cell is killed. This particular locus has been extensively studied, and has conferred nearly absolute stability to ultra-high expression systems that otherwise exhibited very rapid plasmid loss [Wood and Peretti, 1991].

Plasmids and the genes they carry represent a metabolic burden to the cells in which they are maintained. This burden can be divided into two contributions, one involving precursors and energetic molecules, the other involving enzymatic assemblies. Plasmid-bearing (Pb) cells are required to synthesize more DNA, m-RNA, and ribosomes than their Pf counterparts. Thus, Pb cells consume more energy per cell [Collins, 1973]. Additionally, plasmid genes must compete with chromosomal genes for various metabolic precursors (e.g., nucleotides and amino acids) and catalysts involved in protein synthesis, all of which may be present in limiting amounts [Peretti and Bailey, 1987].

One curious observation made regarding cell growth is the influence of growth medium on segregational stability. Godwin and Slater (1979), reported that cells grown in minimal medium exhibited a lower frequency of plasmid loss than did cells grown in rich medium. These results are not explained by growth rate differences between the two cases, but instead hint at the importance of some metabolic precursors/intermediates that are as of yet unidentified. The ratio of growth rates for Pf to Pb cells increases when the growth of the cells is limited by phosphate or magnesium [Klemperer, *et al.*, 1979]. Plasmid stability in continuous cultures increases with decreasing dilution rates; Seo and Bailey (1985), report increased numbers of plasmids per cell and increased expression of the cloned protein with decreasing dilution rate.

Consequently, the turnover rate of Pb cell mass may be less than Pf cells. Classically termed plasmid segregational instability, cultures of Pb cells, unless under selective pressure, will give rise to Pf cells causing a deterioration in culture productivity, especially in continuous reactor systems.

3. Factors Affecting Plasmid Retention and Expression in Immobilized Cultures

Experimental observations of the effects of immobilization, within artificially formed gel bead carriers, on recombinant plasmid stability can be summarized as follows:

1. plasmid vectors exhibit higher retention in the system
2. reduction or elimination of plasmid structural instabilities
3. plasmid copy number maintained or increased
4. operation of the liquid phase of continuous reactors at a residence time less than the generation time of the Pf cells, dilutes them from the system
5. structure of the immobilizing gel separates the two populations, negating competition

6. proximity of immobilized cells less freedom of motion could promote transfer of plasmid DNA between populations by either conjugation (if a mobilizing factor is present) or transformation
7. mass transfer limitations on nutrients may create lower growth rates in the interior of a gel, thus promoting increased plasmid stability

Similar mechanisms may serve to affect plasmid retention in biofilms naturally formed by attaching bacteria. Costerton and co-workers (1992) and Bryers and co-workers (1992) report that biofilms, formed under natural conditions, create highly heterogeneous micro-structures. Use of confocal laser scanning microscopy coupled with fluorescent probe molecules (for Eh, pH, cell viability, cell species number concentration, matrix permeability) clearly indicate adjacent microcolonies within biofilms may see radically different environments. Localized concentrations of calcium may promote plasmid DNA incorporation by transformation. Dense glycocalyx structures may exhibit different mass transfer properties than other sections of biofilm, segregating bacteria into microcolonies much the same way as in gel beads. One distinction between biofilm-bound cells versus artificially immobilized recombinants, is that naturally attaching bacteria must metabolically produce the encapsulating extracellular polysaccharide glycocalyx; an added burden that artificially immobilized cells do not bear. Metabolic effects of biofilm formation and cell replication may affect plasmid segregation differently than in either planktonic or gel-immobilized populations. The effects of polysaccharide synthesis on membrane integrity could influence both conjugation and transformation of plasmid DNA.

Another factor influencing plasmid retention and transfer in a biofilm is the continual intraphase transport of cells at the biofilm-fluid interface; biofilms can be re-inoculated with specific community members which is not likely in gel bead systems. Rochell *et al.* (1989), cultivate two donor *Pseudomonas spp.* with two recipient *Pseudomonas spp.* within biofilms in a laboratory reactor system. All experiments indicated recipient strains survived within the biofilm but not in free suspension. Transconjugants were detected in all cases despite the fact that donor and recipient cells were inoculated at opposing ends of the substratum used. The authors attributed this observation to cell movement from the biofilm to the liquid phase and re-deposition.

Clearly, altered plasmid retention in immobilized or biofilm populations of recombinant cells cannot be explained by any single mechanism. Research has yet to focus on developing methods and systems to assess those environmental and biological factors governing plasmid segregation stability in biofilm populations. Preliminary work on the effects of biofilm growth on plasmid loss due to segregation will be presented below.

4. Mathematical Considerations

4.1 BATCH SUSPENDED CULTURE

In the growth of a culture containing two populations, one containing plasmids X^+ and one having lost the plasmid due to segregational instability, X^-, the dynamics of each population in batch culture can be represented by the following "reaction"-like equations,

$$X^+ \rightarrow (2 - p)\, X^+ + pX^- \qquad \text{rate} = \mu^+ X^+$$

$$X^- \rightarrow 2X^- \qquad \text{rate} = \mu^- X^-$$

where p = number of plasmid-free cells formed per generation of plasmid-bearing cells; in essence the probability of plasmid loss. Imanaka and Aiba (1981) postulate the fraction of cells bearing plasmids, F, after N number of generations of plasmid-bearing cells can be described by

$$F = (1 - \alpha - p) \; / \; [1 - \alpha - p(2^{N(\alpha + p - 1)})] \tag{1}$$

where $\alpha = \mu^- / \mu^+$. There are a number of assumptions tacitly associated with this equation that do not accurately reflect experimental reality. Consequently, an alternative approach to calculating p is proposed.

The population dynamics of suspended plasmid-bearing, X^+, and plasmid-free, X^-, cells in a batch culture can be described by cell growth and plasmid loss [Ollis, 1982],

$$\frac{dX^+}{dt} = \mu^+ X^+ - p\mu^+ X^+ \tag{2}$$

$$\frac{dX^-}{dt} = \mu^- X^- + p\mu^+ X^+ \tag{3}$$

Let $\chi = X^- / X^+$ and substituting χ into Eqn (3),

$$\frac{dX^-}{dt} = X^+ \frac{d\chi}{dt} + \chi \frac{dX^+}{dt} \tag{4}$$

Substituting Eqn (2) and (3) into (4) and rearranging yields,

$$\frac{d\chi}{dt} = (\mu^- - \mu^+ + p\mu^+)\chi + p\mu^+ \tag{5}$$

At exponential phase, $\mu^- = \mu_m^-$ and $\mu^+ = \mu_m^+$. Therefore, Eqn. (2) and (5) can be rewritten as,

$$\frac{dX^+}{dt} = \mu_m^+ X^+ - p\mu_m^+ X^+ \tag{6}$$

$$\frac{d\chi}{dt} = \left(\mu_m^- - \mu_m^+ + p\mu_m^+\right)\chi + p\mu_m^+ \tag{7}$$

Plotting $\ln X^+$ versus time and $d\chi / dt$ versus χ, the resultant slopes and intercept are defined as,

$$m^+ = \mu_m^+ - p\mu_m^+ \tag{8}$$

$$m = \mu_m^- - \mu_m^+ + p\mu_m^+ \quad (9)$$

$$b = p\mu_m^+ \quad (10)$$

Solving Eqns. (8), (9) and (10), $\mu^+{}_m = (m^+ + b)$, $\mu^-{}_m = (m^+ + m)$, and $p = (b / (m^+ + b)$.

4.2 CONTINUOUS BIOFILM CULTURE

Net accumulation of a biofilm culture on a substratum comprises *(1)* deposition of suspended cells from the liquid phase, *(2)* cell replication and extracellular polymer production within biofilm, and *(3)* biofilm detachment due to shear stress [Applegate and Bryers, 1991]; Bryers and Characklis, 1992]. Here, deposition of cells will be ignored since no suspended cells were supplied to the system and suspended cell concentrations in effuent liquid phase were negligible. Therefore, the net accumulation rate of biofilm-bound plasmid-bearing, B^+, and plasmid-free cells, B^-, can be expressed as the combination of cell growth, plasmid loss and detachment,

$$\frac{dB^+}{dt} = \mu^+ B^+ - p\mu^+B^+ - k_{det}B_d^+ \quad (11)$$

$$\frac{dB^-}{dt} = \mu^- B^- + p\mu^+B^+ - k_{det}B_d^- \quad (12)$$

Assuming the spatial distribution of plasmid-bearing and plasmid-free cell within the biofilm are uniform with depth (no spatial gradients of either population), one can define $\beta = B^-/B^+ = B^-_d / B^+_d$ and substitute into Eqn. (12),

$$\frac{dB^-}{dt} = B^+ \frac{d\beta}{dt} + \beta \frac{dB^+}{dt} \quad (13)$$

Substituting Eqn. (11) and (12) into (13) and rearranging . . .

$$\frac{d\beta}{dt} = (\mu^- - \mu^+ + p\,\mu^+)\beta + p\,\mu^+ \quad (14)$$

The specific growth rate of either population can be expressed as,

$$\mu^+ = \frac{\mu_m^+ S}{K_S + S} \quad \text{and} \quad \mu^- = \frac{\mu_m^- S}{K_S + S}$$

Eqn. (14) can now be rewritten as:

$$\frac{d\beta}{dt} = \left(\frac{\mu_m^- S}{K_S + S} - \frac{\mu_m^+ S}{K_S + S} + p \frac{\mu_m^+ S}{K_S + S} \right) \beta + p \frac{\mu_m^+ S}{K_S + S} \quad (15)$$

Plotting dβ/dt versus β, the slope and intercept will be . . .

$$m' = \left(\mu_m^- - \mu_m^+ + p\mu_m^+ \right) \frac{S}{K_S + S} \qquad (16)$$

$$b' = p \frac{\mu_m^+ S}{K_S + S} \qquad (17)$$

Solving Eqn. (16) and (17), one can define a probability of plasmid loss for the biofilm populations as,

$$p = \frac{b'}{m' - b'} \quad \frac{\mu_m^- - \mu_m^+}{\mu_m^+} \qquad (18)$$

5. Experimental Protocol

5.1 BACTERIAL STRAIN AND PLASMID

Escherichia coli DH5α (donated by Dr. Victors Burdett, Department of Microbiology and Immunology, Duke University) was selected as study organism since it can form a biofilm efficiently under low organic carbon substrate concentration and does not produce β-galactosidase. Its genotype is *ø80dlacZΔM15, Δ(lacZYA-argF), U169, deoR, recA1, endA1, hsdR17, supE44, thi-1, gyrA96* (nalidixic acid resistant), *relA1*. Plasmid pMJR1750 is a 7.5-kb plasmid comprising an ampicillin resistant marker, a strong promoter, tac, a repressor gene, *lacI*^{Q}, and *lacZ* gene which encodes for β-galactosidase. Expression of β-galactosidase can be induced by a number of inducers, including isopropyl 13-D-thiogalactoside (IPTG). Cells harboring the plasmid and producing β-galactosidase form blue colonies on agar media containing 5-bromo-4-chloro-3-indol-β-D-galato-pyranoside (X-gal), whereas plasmidfree cells form white colonies.

5.2 BATCH SUSPENDED CELL CULTURE

Fermentation media used was M9 minimal media containing 0.2% glucose, 0.4% casamino acid, and 0.01% thiamine. Batch suspended cultures were carried out in the absence of ampicillin in fully instrumented fermenter of 1 L working volume, at pH 7.0, 37 °C, and with dissolved oxygen maintained above 50% of saturation. 1% (v/v) of exponentially growing cells in identical medium was inoculated to minimize the lag phase. A 10 mL cell suspension was sampled every hour for analysis. For induction experiments, IPTG was added when the suspension absorbance at 600 nm reached 0.8.

5.3 CONTINUOUS BIOFILM FORMATION SYSTEM

Biofilms of *E. coli* DH5α (pMJR1750) were cultivated in a parallel-plate flow cell reactor constructed of optically clear polymethylmethacrylate. Reactor design and

pretreatment are detailed elsewhere [Huang, *et al.* (1992)]. Inoculum was centrifuged from overnight cultures (which were selectively cultivated under 100 µlg/mL ampicillin) and resuspended to 10^8 cells/mL in sterileM9 minimal medium. The flow cell reactor was inoculated by recirculating the cell suspension through the reactor for 2 hours at a flow rate of 45 mL/min. After two hours, the suspension was removed from the recycle loop, a small mixing vessel (100 mL) installed within the loop, the system rinsed and filled with fresh M9 minimal medium supplemented with 50 mg/L glucose, 100 mg/l casamino acid, and 25 mg/L thiamine, and the recirculation flow resumed. Fresh nutrient solution was then delivered to the mixing vessel to affect an overall system dilution rate of 4 h^{-1}. Contents of the mixing vessel were oxygenated with pure oxygen to prevent oxygen limitation. System dilution rate was maintained well in excess of the maximum growth rate of *E. coli* DH5α which served to minimize cell growth in the fluid phase. The mixing vessel and connecting tubing were replaced with sterilized versions, every 12 hours to minimize the biofilm growth outside the flow cell. A micro-dissolved oxygen probe was connected within the loop to measure the dissolved oxygen of the fluid into and leaving the flow cell. Glucose samples were collected and assayed enzymatically every 12 hours. Slides with accumulated biofilm were removed from the flow cell reactor every 12 hours and replaced with clean slides.

Biofilm on a removed slide was scraped completely into 50 mL autoclaved M9 minimal medium and vortexed at maximum for 5 min. to prevent bacterial aggregation. The biofilm suspension was used for the analyses below.

5.4 MEASUREMENT OF PLASMID STABILITY

Segregational instability was determined by LB agar plates that contain 40 µg/mL X-Gal, 40, µg/mL IPTG and 50, µg/mL nalidixic acid. Biomass samples from batch and biofilm cultures were suitably diluted with sterile M9 minimal medium and spread on the plates to form between 30 and 300 colonies for each plate. The number of viable plasmid-bearing and plasmidfree bacteria were determined by averaging the blue and white colonies respectively on three plates. The probability of plasmid loss per cell division was calculated with Eqn. (9) for suspended and the alternative approach described in this paper for biofilm populations. Time derivatives of the ratio of plasmid-free to plasmid-bearing cells for planktonic and biofilm populations, respectively, ($d\chi/dt$ and $d\beta/dt$) in Eqn. (6) and (4) were calculated from a leastsquares, second-order polynomial fit. Structural stability of the plasmid was checked periodically by horizontal gel electrophoresis; no plasmid structural modification was found throughout any experiments.

5.5 β-GALACTOSIDASE ASSAY

Samples obtained from batch and biofilm cultures were centrifuged at 1,000g for 15 min at 5°C. Cell pellets were resuspended in 1 mL TEP buffer (10 mM Tris; 1 mM EDTA, pH 8.0;1 mM PMSF) and disrupted using two 30 sec pulses by Kontes Micro-Ultrasonic Cell Disrupter (Vineland, New Jersey) set at 30% output. The sonicated cellular homogenate was transferred to microcentrifuge tubes and chilled on ice for 10 min, then centrifuged at 5,000g for 10 min to pellet cell debris. β-galactosidase activity on the supernatant was determined by the rate of hydrolysis of the colorless compound, o-nitrophenyl-β-D-galactoside (ONPG), to the yellow chromophore, o-nitrophenol (ONP). 200 µL cell extract was mixed with 2.5 mL reagent A (0.1 M Na_2HP0_4, adjusted to pH 7.3 with O.lM NaH2P04), 100, µL reagent B (3.6M,

β-mercaptoethanol), 100 μL reagent C (30mM MgC12), and 200 IlL reagent D (33.2 mM ONPG in reagent A) in a disposable cuvette. After vortex mixing, the cuvette was placed in a UV/VIS spectrophotometer and absorbance changes read at 410 nm over a two-minute interval. The activity was calculated using Beer's law and one unit of, β-galactosidase was defined as the amount of enzyme that can hydrolyze 1 μmol ONPG in one min at pH 7.3 and 25°C. The amount of β-galactosidase was determined by calibrating the activities of aliquots of standard, β-galactosidase solution (5 Prime → 3 Prime, Inc., Boulder, Colorado).

6. Results

6.1 PLASMID SEGREGATIONAL INSTABILITY

The probability of plasmid loss for E. *coli* DH5α (pMJR1750) was determined from the relative distribution of plasmid-free and plasmid-bearing cells for cultures grown either in a free suspension batch reactor (Figure l) or within a biofilm accumulating within a CSTR (Figure 2). In suspension, one can directly see that the plasmid-free cell population grows at a rate that allows the plasmid-free population to rapidly dominate the culture in the absence of antibiotic. The probabilities of plasmid loss were calculated for the suspended cultures based on Eqns. (6) and (9) and are summarized in Table 2 for two alternative methods of estimation; the classic plasmid loss probability calculation [Huang, *et al.*, 1992] and the model presented here. Respectively, the classic method predicts a p = 0.0009 while Eqns. (6) and (9) predict = 0.0002.

The difference in the plasmid loss probabilities estimated by the two methods can be attributed to *(1)* Imanaka and Aiba (1981) assumed their initial plasmid-free cell concentration to be zero when in practicality this is not likely and *(2)* the estimation is very sensitive to the value of generation number used which was different in the two studies.

Table 2. Parameters And probabilities for suspended batch culture of *E. coli* DH5α / pMJR1750.

	Imanaka and Aiba (1981)				This Study			
Expt.	n[a]	F(n)[a]	α	p[b]	m+	m	b	p
A	8	0.9793	1.17	0.0023	0.41	0.07	0.0004	0.0010
B	9	0.9747	1.25	0.0017	0.52	0.13	0.0003	0.0006
C	8	0.9774	1.15	0.0027	0.41	0.06	0.0005	0.0012
Ave.				0.0022				0.0009

a: Determined by the final data point of experiment
b: Solved by the ZREAL subroutine from IMSL mathematical library

Since the rate of cell concentration change in a biofilm is the net result of a number of processes, only one of which is cell growth, no interpretation can be made regarding the growth rate of the two populations in the biofilm directly from the data. However, Figure 2 does indicate that plasmid-free cells do "accumulate" faster in the biofilm versus plasmid-bearing cells. However, this observation could be the result of either a preferentially higher erosion rate of plasmid-bearing cells causing from the

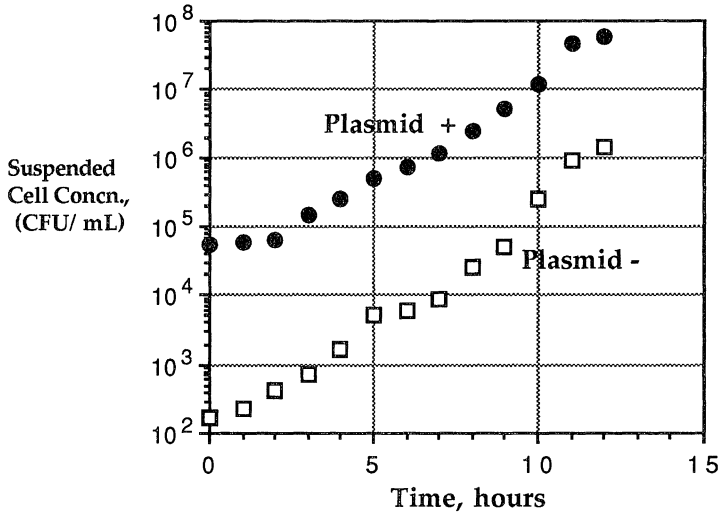


Figure 1. Suspended cell concentrations of plasmid-bearing (●) and plasmid-free (❑) *E. coli* DH5α (pMJR1750) in batch culture without antibiotic selection.

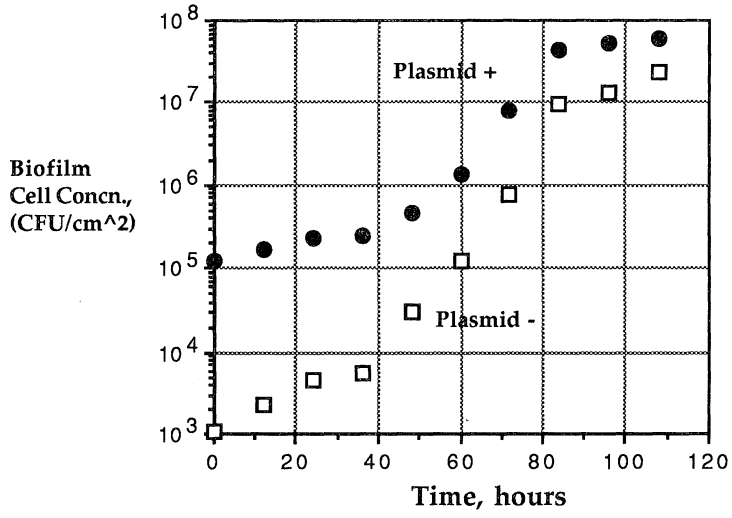


Figure 2. Net accumulation of plasmid-bearing (●) and plasmid-free (❑) *E. coli* DH5α (pMJR1750) in biofilm continuous culture without antibiotic selection.

upper layers of the biofilm or substrate mass transfer limitations on slower growing plasmid-bearing cells be relegated to the depths of the biofilm. Using Eqns. (14) to (17) with the average μ^+_m and , μ^-_m from batch suspended cultures, plasmid loss probabilities (Table 3) for the biofilm populations ranged from 0.013 to 0.021 over a number of experiments, with the mean value being 0.017.

Table 3. Parameters and probabilities for biofilm culture of *E. coli* DH5α / pMJR1750.

Experiment	Sticking Efficiency	m'	b'	p
A	2.0×10^{-7}	0.0187	0.0015	0.013
B	1.92×10^{-7}	0.0058	0.0007	0.021
C	3.50×10^{-7}	0.0115	0.0012	0.018
Average	2.50×10^{-7}			0.017

6.2 β-GALACTOSIDASE EXPRESSION AND EFFECTS OF INDUCER

The effects of different concentration of the inducer IPTG on the activity of enzyme β-galactosidase were determined on cells in a batch suspended and continuous biofilm cultures are shown, respectively, in Figures 3 and 4. Maximum growth rate of cells in batch culture without IPTG added reached 0.52 h^{-1} but once the inducer was added, growth drops accordingly. At IPTG concentrations under 0.17 mM the grow rate is 0.35 h^{-1}. Under 0.34 and 0.51 mM IPTG, growth rates of the culture dropped to 0.16 h^{-1}. At IPTG concentrations less 0.17 mM, maximum, β-galactosidase concentrations during the experiment were 0.32 pg/cell. Under 0.34 and 0.51 mM IPTG, respectively, β-galactosidase concentrations peaked at 0.47 pg/cell. Once induced, the plasmid bearing cells rapidly decreased in population concentration upon expression of the protein. Figure 3B illustrates no significant effect of varying IPTG levels on the growth rate of plasmid-free E. *coli* DH5α.

Figure 4A and 4B illustrate similar population shifts in a biofilm community brought on by induced recombinant protein expression during the addition of similar amounts of IPTG. The question is whether the loss rate of plasmids immobilized is greater or less in a biofilm than in suspension? Again, it would convenient to argue that plasmid-bearing cells decrease rapidly upon induction since induction brings on slow growth. However, this may not be entirely true since cells populations in the biofilm are governed by processes other than growth. Maximum β-galactosidase concentrations produced upon induction were 0.08, 0.10, and 0.12 pg/cell at 0.17, 0.34, and 0.51 mM IPTG, respectively; more than 70% less protein per cell than freely suspended.

7. Concluding Remarks

Contrary to reports of improved segregational stability of plasmids in gel-immobilized systems, calculations here indicate, without induction, the probability of loss of plasmid pMJR1750 is higher in a biofilm than in suspension. Probability of plasmid loss can be affected by multiple factors including copy number, medium, composition, and growth rates. While the medium composition to the two systems

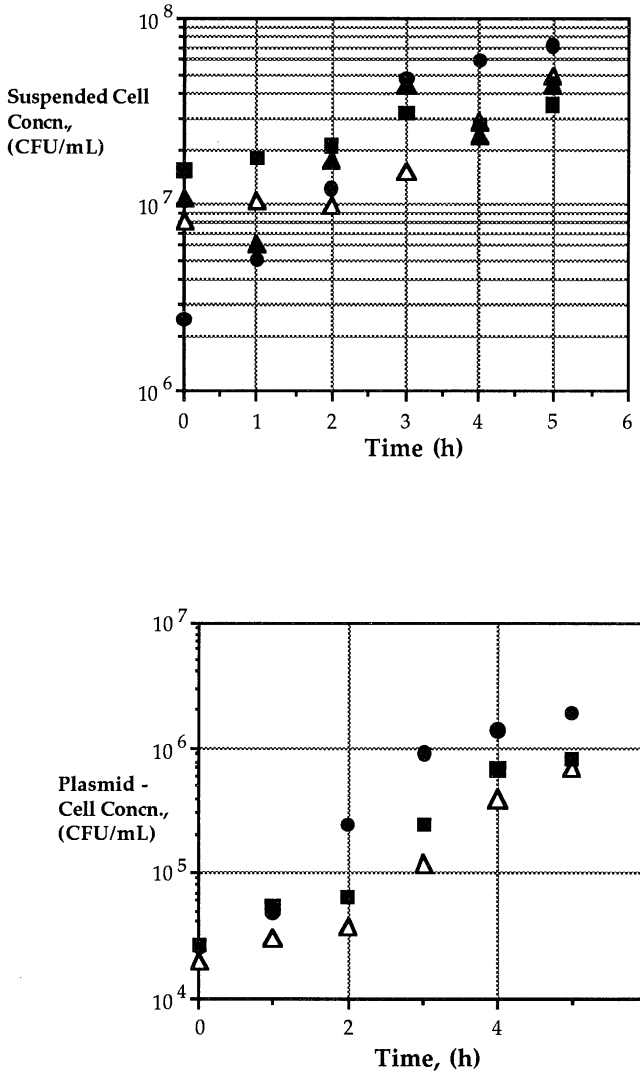
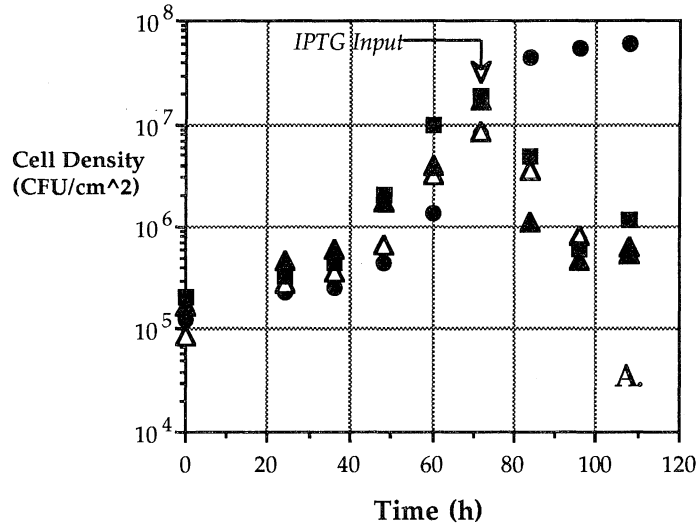


Figure 3. Effects of IPTG induction on the rate of change of both plasmid-bearing (**A.**) and plasmid-free (**B.**) *E. coli* DH5α (pMJR1750) in batch suspended cultures. IPTG Amounts (in mM):(○) = 0, (Δ) = 0.17, (■) = 0.34, and (▲) = 0.51.



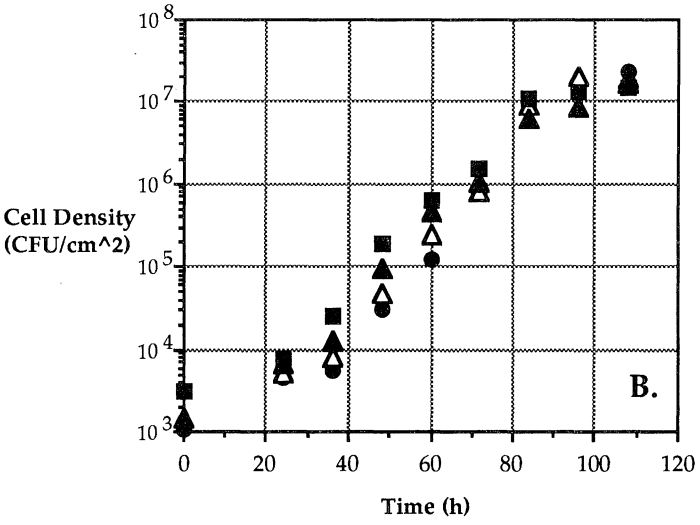


Figure 4. Response of (**A.**) plasmid-bearing, B+, and (**B.**) plasmid-free, B-, *E. coli* DH5α cultivated continuously in a biofilm to a pulse addition of various concentrations of the inducer, IPTG. IPTG Amounts (in mM):(○) = 0, (Δ) = 0.17, (■) = 0.34, and (▲) = 0.51.

was the same, variations in plasmid copy numbers between suspended and biofilm cultures are possible. Growth rates of the suspended culture varies throughout the batch culture, except during exponential phase. At least all cells are exposed to similar liquid phase conditions, while as the biofilm develops mass transfer limitations could affect a gradient of growth rates spatially in the biofilm.

Another possibility for the difference in p values between suspended and biofilm experiments may lie in our simplistic model - i.e., is this observation fact or an artifact of the model employed? The model assumes *(1)* that deposition of suspended cells onto the biofilm does not occur since cells are not supplied to the system and *(2)* that spatial gradients of plasmid-free and plasmid-bearing cells do not develop with depth over time.

The first assumption ignores the possibility of cells detaching from the biofilm at one point in the flow cell then re-attaching at another. Estimates of cell sticking efficiencies (Table 3) at the exit fluid concentration of 105 cells/mL and the mean hydraulic residence time of a cell in the biofilm study reactor suggest re-attachment is negligible.

The second simplification affects the estimate of cell distribution detaching from the biofilm. Our model calculations of loss probability, p, (Table 2) assumed that the B+ and B- cells were uniformly distributed spatially in the biofilm; thus each population would detach from the film: fluid interface at a rate proportional to its average concentration in the entire biofilm. However, if one population's turnover rate was higher than the other, it would eventually dominate the upper layers of the biofilm and would also dominate the mass removed at the biofilm:fluid interface. One can circumstantially determine if stratified biofilm populations develop by comparing the ratios of the different cell types in both the biofilm (a composite parameters averaged over the entire depth of the biofilm) versus cell concentrations in the fluid phase arising due to shear removal. Assuming cells are detached only at the upper layers of the biofilm, with the reactor operated well past wash-out, those cells in the fluid phase would provide an indirect indication of the ecology of only the upper biofilm layers. Figure 5 illustrates the time course of both plasmid-bearing (X+) and plasmidfree (X-) cells suspended in the liquid phase during the accumulation of biofilm illustrated in Figure 2.

Figure 6 compares the ratio of plasmid-free to plasmid-bearing cells in the biofilm, B, to the same ratio determined for biofilm cells detached and re-entrained into the liquid phase, $\beta_{detached}$. Figures 5 and 6 clearly indicate that the relative amounts of B+ and B- cells in the biofilm and detached are approximately the same until the biofilm thickness attains its maximum steady-state value at ~ 48 hours. However, over prolonged growth, the faster growing B- cells begin to outcompete the B+ cells in the upper layers of the biofilm leading to a disproportionate amount of B- cells being sheared off into the liquid. This stratification is also indirectly indicated by the rapid increase in , $\beta_{detached}$ versus , β.

Consequently, assuming a spatially uniform biofilm may lead to erroneous estimates of plasmid loss probabilities. We are now employing a more sophisticated model to account for spatial distributions in our estimates of plasmid stability as well as employing a modified plasmid pMJR1750 which incorporates a suicidal stabilizing *parB* locus. This later revision will eliminate the generation of plasmid-free cells during a culture. Differences in plasmid retention in systems with and without the *parB* locus will allow estimates of potential mechanisms of plasmid stability such as conjugation or transformation.

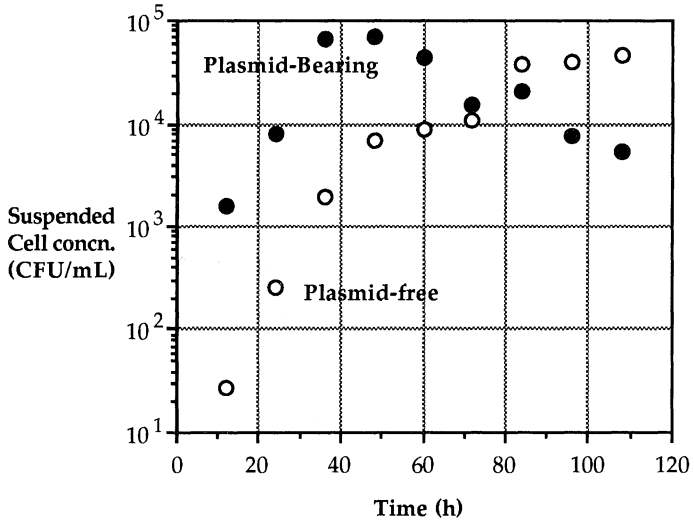


Figure 5. Concentration of plasmid-bearing (●) and plasmid-free (○) *E. coli* DH5α detached from a developing biofilm (ref. Figure 2) and re-suspended in the reactor liquid phase.

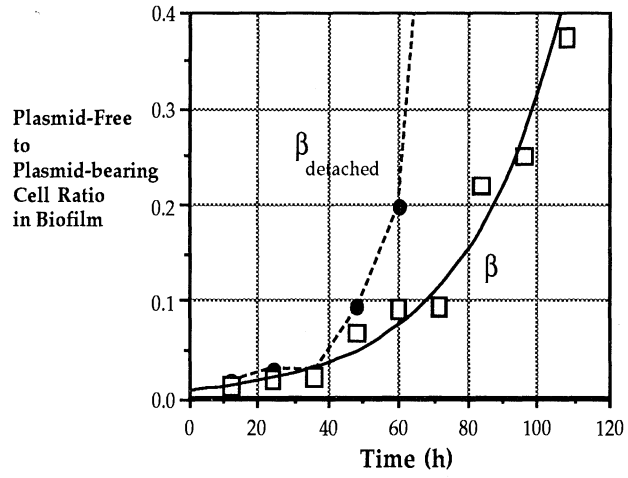


Figure 6. Ratio of plasmid-free to plasmid-bearing *E. coli* DH5α (pMJR1705) cells in a developing biofilm, β, (□) and in the cells detached from the biofilm a resuspended in the reactor bulk liquid, $\beta_{detached}$ (●).

8. References

Adams, J., Kinney, T., Thompson, S., Rubin, L., & Helling, R. E. (1979) *Genetics, 91, 627-637.*

Applegate, D. H. and Bryers, J. D. (1991) *Biotechnol. Bioengrg.*, 37,17-25.

Bailey, J. E. & Ollis, D. *F.* (1986) *Diochemical Engineering Fundamentals, 2nd Ed.*, McGrawHill, New York.

Bryers, J.D. & Characklis, W.G. (1992) in Melo *et al.* (eds.) Biofilms - Science and Technology, Kluwer Academic Publishers, Dordrecht, The Netherlands, this volume.

Bryers, J. D. & Drummond F. (1992) personal communication, unpublished doctoral research.

Clewell, D. B. (1981) *MIcrobiol. Rev.*, 45, 409.

Clewell, D. B., Yagi, Y., Ike, R.A., Craig, B. L. & Brown, F. (1982) *Microbiology - 1982,* D. Schlesinger, (Ed) ASM, Washington, D.C., 97-100.

Collins, J. & Pritchard, R. H. (1973) *J. Mol. Biol., 78, 143-155.*

Costerton, J. W. (1992) personal communication.

Da Silva, N. A. & Bailey, J. E. (1986) *Biotechnol. Bioengrg., 28,* 741-746.

de Taxis du Poet, P., Dhuster, P., Barbotin, J. -N., & Thomas, D. *(1986) J. Bacteriol., 165, 871-877.*

Doran, P. M. & Bailey, J. E. (1986) *Biotechnol. Bioengrg.*, 28, 73-87.

Doran, P. M. & Bailey, J. E. (1987) *Biotechnol. Bioengrg.*, 29, 892-897.

Dykhuizen, D. E. & Hartl, D. L. *(1983) Microbiol. Reviews, 47,150.* Inloes, D. 5., Smith, W. J., Taylor, D. P. Cohen, 5. N., Michaels, A. 5., & Robertson, C. R. (1983) *Biotechnol. Bioengrg., 25, 2653-2681.*

Engberg, B. & Nordstrom, K. (1975) *J. Bacteriol. ,123,179-186.*

Fredrickson, A. (1977) *Ann. Rev. Microbiol., 31,* 63-87.

Freter, R., Freter, R. R., & Brickner, H., (1983) *Infect. Immunol., 39, 60-84.*

Gealt, M. A., Chai, M. D., Alpert, K.B., & Boyer, J. C. (1985) *Appl. Environ. Microbiol.*, 49, 836-841.

Gerges, K. (1988) *Bio/technology, 6,1402-1405*

Godwin, D. & Slater, J. H. (1979) *J. Gen. Microbiol.*, 111, *201-207.*

Graham, J. B. & Istock, C. A. (1978) *Mol. Gen. Genetics,* 166, 287-290.

Graham, J. B. & Istock, C. A. (1979) *Science,* 637-639.

Grandi, G., Mottes, M., & Sagaramella, V. *(1981) Plasmid, 6, 99.*

Hattori, R. (1972) J. *Gen. Appl. Microbiol.*, 18, 319-330.

Huang, C. -T., Peretti, S. W., and Bryers, J. D. (1992) Plasmid retention and gene expression in batch suspended and continuous biofilm cultures of the recombinant *E. coli* DH5a (pMJR1750), *Biotechnol. Bioengrg.*, submitted for publication, April, 1992.

Imanaka, T. and Aiba, S. (1981) *Annals New York Academy of Sciences,* 369, 1-14.

Inselberg, J. *(1978) J. Bacteriol., 133, 433-436.*

Jones, I. M., Primrose, 5. B., Robinson, A., & Ellwood, D. C. (1980) *Mol. Gen. Genetics, 180, 579-584.*

Kadam, K. L., Wollweber, K. L., Grosch, J. C., & Tao, Y. C. (1987) *Biotechnol. Bioengrg., 29, 859-872.*

Klemperer, R. M. M., Ismail, N. T. A., & Brown, M. R. W. (1979) *J. Gen. Microbiol., 155, 325.*

Kumar, P. K. R. and Schügerl, K. (1990) J. *Biotechnol.*, 14, 255-272.

Lauffenburger, D. A., (1987) *Trends in Biotechnol.*, 5, 87-89.

Levin, B. R., Stewart, F. M. & Rice, V. A. *(1979) Plasmid, 2, 247.*

Meacock, P. A. & Cohen, 5. N. (1980) *Cell, 20, 529-542.*

Meacock, P. A. & Cohen, S. N. (1980) *Cell,* 20, 529-542.

Monbouquette, H. G. & Ollis, D. F. (1988) *Bio/technology,* 6,1076.

Morrison, W. D., Miller, R. V., & Sayler, G. 5., (1978) *Appl. Environ. Microbiol., 44, 13951403.*
Nakazawa, T. (1978) *J. Bacteriol., 133, 527-535.*
Nasri, M., Sayadi, 5., Barbotin, J. -N., Dhuster, P., & Thomas, D. (1987) *Appl. Environ. Microbiol., 53, 740-744,*
Navarro, J. M. & Durand, G. (1977) *Eur. J. Appl. Microbiol.* 4, 243-254.
Ollis, D. F., (1982) *Phil. Trans. Royal Soc., London,* B, *297,* 617-629.
Ou, L. -T. & Alexander, M. (1974) *Arch. Microbiol.,* 101, 35-44.
Peretti, S. W. and Bailey, J. E. (1987) *Biotechnol. Bioengrg., 29,* 316-325.
Peretti, S. W., Bailey, J. E., & Lee, J. J. (1989) *Biotechnol. Bioengrg., 32.*
Reanney, D. C., Roberts, W. P., & Kelly, W. J. (1982) *Microbial Interactions and Communities,* Vol. I, A. T. Bull & J. H. Slater (Eds.), Academic Press, Inc., New York.
Rochell, P. A., Fry, J C. and Day, M. J. (1989) *rEMS Microbiol. Ecology,* 62, 127-136.
Saye, D. J., Ogunseiten, 0., Sayler, G. 5., & Miller, R. V., *(1987) Appl. Environ. Microbiol., 53, 987-995.*
Seo, J. -H. & Bailey, J. E. (1985) *Biotechnol. Bioengrg., 27,* 1668-1673.
Skogman, 5. *(1983) Gene, 23,105-115.*
Stewart, G. J., & Carlson, C. A. (1986) *Ann. Rev. Microbiol.,* 40, 211-235.
Stewart, G. J., Carlson, C. A., & Ingraham, J. L. (1983) J. *Bacteriol.,* 156, 30-35.
Tyagi, R. D. & Ghose, T. K. (1982) *Biotechnol. Bioengrg.,* 24, 781-796.
Uhlin, B. E. & Nordstrom, K. (1978) *Mol. Gen. Genetics,* 165,167-179.
Uhlin, B. E., Molin, S., Gustafsson, P., & Nordstrom, K. (1979) *Gene,* 6, 91-106.
Weber, A. E. and K. -Y. San, (1987) Biotechnol. Letters, 9, 757.
Weinberg, S. R., & Stosky, G. (1972) *Soil Biol. Biochem.,* 4, 171-180.
Wood, T. K. & Peretti, 5. W. (1990) *Biotechnol. Techni~ues, 40, 39-44.*
Wood, T. K. and Peretti, S. W. (1991) *Biotechnol. Bioengrg.,* 38, 397-412.
Wouters, J. T., Driehus, F. L., Polaczek, P. J., van Oppenraag, M. L. & van Andel, J. G. (1980) *Ant. van Leeuwen.,* 46, 353.
Zeph, L. R., Onaga, M. A., & Stozky, G. (1988) *Appl. Environ. Microbiol.,* 54, 731-1737.
Zund, P. & Lebek, G. (1980) *Plasmid, 3,* 65-69.

PARTICLE-BACTERIA INTERACTIONS IN BIOFILMS

T.R. BOTT
University of Birmingham
School of Chemical Engineering
Birmingham B15 2TT, United Kingdom

L.F. MELO
University of Minho
Biological Engineering
4700 Braga, Portugal

1. Introduction

Water is used extensively for cooling purposes in a wide variety of industrial processes and a major problem in efficient operation is the presence of biofilms on the surface of equipment. On the other hand, in wastewater treatment, the development of a robust biofilm is essential for the effectiveness of the process.

Industrial cooling water is generally obtained from one of several sources, such as towns water, bore holes, lakes, reservoirs, rivers or canals. The former is costly and may only be used in special or limited circumstances. Bore holes may be employed if the particular site has suitable geological features. Sea water may also be used where the process plant is conveniently placed. For large cooling systems, e.g. power station condensers, river water is often used because of the plentiful supply at low cost. The use of river water not only introduces microorganisms but also particulate matter.

Common particulate material includes sand, silt, clay or quartz. To a lesser extent, metal oxides, resulting from the corrosion of industrial equipment surfaces may also be present within heat exchangers, cooling towers, bioreactors, etc.

In general, water from bore holes will contain low concentrations of suspended solids, i.e., < 5 mg/l. On the other hand, the concentration of suspended particles in river water is likely to vary according to the local geography, the climatic conditions and the quantity of the water in the course. Work by Mansfield (1983) on a river in the North of England shows large variations in the particulate concentration, but in general the level was less than 5 mg/l for 50% of the time. Rhine river water analysed by Novak (1981) contained 5-17 mg/l of total suspended material. Particulate matter concentrations measured by Teixeira and Silva (1990) in a river in the north of Portugal ranged between 10 mg/l and 65 mg/l. At a particular site the level of sand particles in sea water was in the range 50-5000 mg/l (Parker and Roscow, 1981).

The study of river water conducted by Mansfield (1983) revealed that the suspended particle diameter was generally in the range 0-15 μm with two peak concentrations at 1-2 μm and 5-9 μm. Larger particles are likely to be suspended if the river flow is increased, i.e., after heavy rain or melting snow.

Suspended particle concentrations can be high in wastewaters from several industries (before treatment): 150 mg/l in effluents from textile industries (Teixeira, 1988); 200 mg/l in process water used in an oil refinery (Urbano and Fernandes, 1988). A wide variety of suspended particles can be found in wastewaters, from clays and iron oxides to fibers (e.g., cellulose, leather), insoluble waxes and colloidal pigments, depending on the type of industry. Sometimes, the small size of such particles reduces the possibility of eliminating them by filtration. The efficiency of

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 199–206.

biofilm reactors used in effluent treatment will then be affected by the presence of the particulate material, which has to be degraded (hydrolysed) before the "clean" liquid is dicharged from the reactor.

2. Cell-Particle Interactions

Some work has been published on the interactions between simple microorganisms and particles: Stotzky (1966); Burns (1979); Filip and Hattori (1984); Hoppe (1984), among others. Few data were published on the effects of particulate material on biofilms formed in simulated cooling water systems (Lowe, Duddridge, Pritchard and Bott, 1984; Pinheiro, Melo, Pinheiro and Bott, 1988; Oliveira, Vieira, Melo and Pinheiro, 1990) and on biofilms used in effluent treatment processes (Särner, 1986; Bouwer, 1987).

The interaction between microorganisms and suspended particles is likely to have a pronounced effect on the character and extent of biofilms adhering to surfaces in contact with flowing water or wastewater. Threee effects may be envisaged:

1) The effect of particulate matter on the availability of nutrients to the microorganisms and the effect on their metabolism. The influence of solid adsorbents on microbial adhesion and metabolic activity was studied by Filip and Hattori (1984) and by Hoppe (1984). In general, the result was an increase in the specific growth rate and in the biological activity. Clay minerals, such as montmorillonite, were shown to enchance biomass formation in aerobic cultures of *Saccharomices cerevisiae*, but not in anaerobic cultures (Filip and Hattori, 1984). Glucose consumption increased in the presence of montmorillonite under aerobic conditions, as well as ethanol production by unit mass of consumed glucose. This effect was seen as the result of the adsorption of metabolic inhibitors by the clay particles. Higher growth rates of *Aeromonas sp.* were found in the presence of kaolin particles (Hoppe, 1988) and were explained by "physiological changes" in the attached cells. Stotzky (1966) concluded that clay minerals stimulated bacterial respiration by removing or neutralizing acidic metabolites that decrease the pH to an inhibitory level. Burns (1979) pointed out that the adsorption characteristics of clays are particularly high due to their ability to expand in water, increasing the available surface area (external and internal).

However, in different conditions, chemical activities of non-growing cells of *Pseudomonas fluorescens*, *E. coli* and others were reduced by the adhesion to resin surfaces (Filip and Hattori, 1984).

To explain the effect of kaolin particles on biofilm formation by Pseudomonas fluorescens in turbulent flow situations, it has been suggested (Pinheiro et al., 1984; Oliveira et al., 1990) that kaolin particles adsorb substrate molecules into their surface that help to concentrate nutrients to the benefits of growing bacteria. This effect would be particularly significant when particles become incorporated in the biofilm, i.e., providing additional nutrient transport to the microorganisms residing in the biofilm on the surface, over and above that provided by natural diffusion.

2) The erosion effects of the particles that leads to the removal or suppression of biofilm formation. It has been suggested by Bour and Battaglia (1981) that low levels of biofouling experienced in condensers are due to the scouring effect of solid particles.

3) The presence of the biofilm enhances the capture of particulate matter from the flowing systems that increases the accumulation of material on surfaces.

3. Simulated Cooling Water Systems

Studies of laboratory cooling water systems have been made by Lowe (1988), Pinheiro (1987) and Vieira (1992) and some data published (Lowe et al., 1984; Pinheiro et al., 1988; Oliveira et al., 1990). Although the work was carried out in two different laboratories, the principle of the experimental apparatus used in the studies is the same.

In order to facilitate consistent operation of the system, a single microorganism *Pseudomonas fluorescens* was grown under carefully controlled conditions in a laboratory fermenter. Once the fermenter had reached steady state, it was possible to draw off a constant supply of bacteria that could be introduced continuously in the circulation loop. The procedure ensured a constant concentration of cells in the flow system. A suspension of kaolin and a nutrient stream were added to the circulating system so that a fixed concentration of kaolin was maintained together with a constant concentration of substrate assessed in terms of glucose concentration. Kaolin was choosen as the suspended solid since it represents clay particles that might be encountered in an industrial cooling water. In order to maintain concentrations and to allow for the addition of fresh water in the various streams, a constant bleed of water ensured constant conditions within the system. All solutions and suspensions entering the circulation loop were sterilised before use and the fermenter was operated aseptically, apart from the inoculum of the test bacteria.

The velocity, temperature and pH of the circulating water were monitored and controlled. Specially designed test sections allowed the decay in heat transfer coefficient to be observed or the accumulation of deposit to be measured. The test sections were mounted in the vertical position to avoid gravitational effects. Details of the experimental conditions have been described (Lowe et al., 1984; Oliveira et al., 1990) and are summarised in Table 1.

TABLE 1: Summary of experimental conditions.

	Lowe (1988)	Vieira (1992)
Microbial cell concentration	10^7 cells/ml	6.10^7 cells/ml
Kaolin concentration	5 mg/l	150 mg/l
Original kaolin particle size	2 - 5 μm	12 μm
Glucose supplement to give	2 mg/l	20 mg/l
pH of circulation water	7	6.5 - 7
Recirculation residence time	20 minutes	70 minutes
Temperature	25°C	27°C
Flow velocities	1.5, 1.2, 0.5 m/s	0.9, 0.6, 0.3 m/s
Test sections	Tubes 15.6 mm internal diameter, 576 mm long fabricated from 316 stainless steel	Semi-circular ducts 18 mm hydraulic diameter, 1m long, made of aluminium
Physical property measured to assess the amount of biofilm	Weight	Thermal resistance

3.1 DATA FROM LOWE (1988)

The amount of biofilm accumulation in Lowe's work was assessed by weighing the tube before and after the appropriate time lapse, as explained elsewhere (Lowe et al., 1984). Data obtained in these tests are summarised in Table 2. The data demonstrate that at the low kaolin concentration of 5 mg/l there is little difference in the mass of biofilm obtained when compared with identical operating conditions with no kaolin present.

TABLE 2: Data on maximum biofilm wet mass (Lowe, 1988).

Velocity (m/s)	Maximum wet mass ($g/cm^2 \times 10^{-4}$)		Approximate time to achieve maximum wet mass (hours)	
	5 mg/l kaolin in suspension	No kaolin in suspension	5 mg/l kaolin in suspension	No kaolin in suspension
0.5	60	105	250	250
1.2	175	105	210	240
1.5	250	300	220	230

It would also appear, from Table 2, that as the water velocity increases the maximum value of the mass of bioflm (plus adhering particles) also increases.

Table 3 provides data on the inorganic and organic content of the biomass, as a percentage of the total wet biofilm. These data were obtained by weighing to a constant mass at 50°C, and the organic content was taken as the difference between this mass and the mass after exposure to air at 500°C in a muffle furnace. The final mass of the residue gave an indication of the inorganic content of the particular biofilm.

TABLE 3: Inorganic and organic content of biofilms containing kaolin (Lowe, 1988).

Velocity flow (m/s)	Inorganic content as % of wet biofilm mass	Organic content as % of wet biofouling mass
0.5	4.54	1.21
1.2	6.26	1.38
1.5	6.25	1.47

The increase in biofilm mass with the fluid velocity is accompanied by a similar increase in the fraction of inorganic particles in the biofilm. Therefore, in spite of the higher water velocities, the presence of a larger fraction of kaolin particles seems to reinforce the deposit structure against the shear stress effects.

Experiments were conducted to investigate the scouring effects of both kaolin and sand at different surface shear stress in comparison to the removal effects of water alone. Lowe showed that there was little difference between removal achieved by kaolin in suspension in concentrations of 50, 1000 and 5000 mg/l and water alone on established biofilm. At a shear stress (calculated from the Blasius equation) of 75 N/m^2, approximately 75% of the original biofilm had been removed in both cases.

For a sand suspension of 5000 mg/l and above, there are indications of enhanced removal compared to water alone. At around 75 N/m^2 shear stress, approximately 90% of the biofilm was removed for fine sand suspensions of 5000 and 20000 mg/l. A suspension of coarse sand at a concentration of 20000 mg/l removed 95% of the original biofilm.

3.2 DATA FROM VIEIRA (1992)

Table 4 indicates the maximum (asymptotic) thermal resistances of the biofilms obtained by Vieira (1992), with and without kaolin in suspension. Reynolds numbers varied between 3500 and 10000, approximately.

TABLE 4: Asymptotic thermal resistance of biofilm (Vieira, 1992).

Fluid velocity (m/s)	Maximum thermal resistance of biofilm (after 10 days) ($m^2.K/W \times 10^{-4}$)	
	150 mg/l of kaolin in suspension	No kaolin in suspension
0.28	42	----
0.34	----	42
0.54	----	31
0.59	32	----
0.82	----	10
0.87	20	----
1.24	7.4	3.6

Two conclusions may be drawn: a) the maximum thermal resistance of the biofilms decreases with increasing velocity: b) the presence of kaolin enchances biofilm development, although this effect is more clear for higher velocities.

It should be stressed that the thermal conductivity of kaolin particles is lower than that of the pure biofilm, which could explain the higher thermal resistances of the mixed biofilm. To test this argument, some experiments were carried out where nutrients were removed from the fluid once the deposit thermal resistance reached its maximum value.

TABLE 5: Effect of nutrient exclusion on biofilm development (Vieira, 1992).

Fluid velocity (m/s)	Interval from the time the nutrients were excluded to the time the biofilm began to collapse	
	150 mg/l of kaolin in suspension	No kaolin in suspension
0.34	2.5 days	2 days
0.52	----	1 day
0.59	2.5 days	----
0.71	2.0 days	1 day
0.93	2.5 days	1 day

Table 5 shows that some time after the exclusion of the nutrients the amount of deposit started to decrease. However, the mixed deposit (bacteria + kaolin) maintained its structure intact for a longer period than the biofilm containing only bacteria. This suggests that the mixed deposit contains a higher amount of nutrients which enables the bacteria to survive for a longer period after the substrate has been removed from the suspension. An explanation could be that the kaolin particles may act as reservoirs of nutrients in the biofilm, or that the deposit has a different structure which facilitates the internal diffusion of nutrients.

Once more, the differences between the two types of biofilms can be more clearly detected for higher than for lower water velocities. In fact, higher velocities result in higher diffusion rates of substrate and particles towards the biofilm which may contribute to a higher availability of nutrients in the inner layers of the deposit.

3.3 COMPARISON BETWEEN THE RESULTS OF LOWE AND VIEIRA

Altough the two sets of results were obtained in similar rigs, the operating conditions were different, mainly as regards the concentrations of nutrients and kaolin in the suspension. A striking difference is the effect of fluid velocity on the amount of biofilm formed. Lowe's experiments were made with a much smaller concentration of nutrients leading to much thinner biofilms than in Vieira's tests. Taking, for instance, the biofilm obtained by Vieira with the fluid flowing at 0.59 m/s, and considering that the values for biofilm density and thermal conductivity are not far from those of the water (Characklis, 1981), the mass of biofilm corresponding to the thermal resistance of 32.10^{-4} m^2 K/W will be 0.19 g/cm^2. This is 32 times greater than the biofilm mass obtained by Lowe (0.006 g/cm^2) at the velocity of 0.5 m/s.

It is well known that the shear effects of the liquid usually increase as the thickness of the deposits increase. Therefore, these effects would be much less pronounced in the tests performed by Lowe and even less if it is assumed that the kaolin particles tend to reinforce the structure of the biofilm. The data shown in Table 3 support this hypothesis, since the percentage of inorganic particles in the deposit increases with the fluid velocity.

On the other hand, the biofilms produced by Vieira are much thicker and less resistant to the shear stress, their upper layers being easily removed by the water flow - in such a case, the higher the velocity the thinner the deposit will be.

Another difference between the two sets of results is the fact that the presence of kaolin appears to favour the development of the biofilm in Vieira's experiments, but not in Lowe's. Surely, the effect of kaolin must be much more "visible" if large concentrations of particles are used, as in the work of Vieira.

4. Biological Treatment Systems

The effect of particles in effluent biological treatment processes is usually described in terms of the need to hydrolyse the particles (Sarner, 1986; Bouwer, 1987; Olthof and Oleszkiewicz, 1982), little being said about the characteristics and behaviour of the biofilm under such conditions.

Since the time for hydrolysis is greater when particles are present in the effluent, this process may become the rate limiting step for the conversion of substrate. It was stated that the adsorption of organic particles on the biofilm surface decreases the rate of degradation of soluble substrate (Sarner, 1986), since they may cause a shortage of oxygen in those zones. This effect was measured only at high substrate concentration and at reasonably high temperatures, that is when oxygen consumption is highest. Curiously, when low nutrient concentration and low temperatures were used, the opposite effect was observed.

5. Concluding Remarks

It appears, from the literature, that:

1) The amount of data available on particle-bacteria interactions in biofilms is too scarce in order to have a sound opinion on the subject.

2) The effect of suspended particles on biofilm formation is very dependent on the operating conditions and, particularly, on the kind of particles and microorganisms present. The result can be either an enhancement of biofilm growth and stability, or the reverse.

3) Clearly, more work has to be done, both in cooling water and in effluent treatment systems so that the mechanisms for avoiding or promoting biofilm formation may be better understood.

ACKNOWLEDGEMENTS

Much of the experimental data presented were obtained by Martin Lowe and Maria João Vieira, to whom the authors express their gratitude.

REFERENCES

Bouwer, E.J. (1987) 'Theoretical investigation of particle deposition in biofilm systems', Water Research, 21, 1489-1498.

Bour, D.P., Battaglia, P.J. and Burd, K.M. (1981) 'Biofouling control practice and assessment', EPRI Report CS-1796, Pub. N.U.S. Corp., Pittsburgh, USA.

Burns, R.G. (1979) 'Interaction of microorganisms, their substrates and their products with soil surfaces', in Elwood, Melling and Rutter (eds.), Adhesion of Microorganisms to Surfaces, Academic Press, London, pp. 109-138.

Characklis, W.G. (1981) 'Microbial fouling: a process analysis', in Somerscales and Knudsen (eds), Fouling of Heat Transfer Equipment, Hemisphere Publish. Corp., N.Y., pp. 251-291.

Filip, Z. and Hattori, T. (1984) 'Utilization of substrates and transformation of solid substrata', in K.C. Marshall (ed.), Microbial Adhesion and Aggregation, Springer-Verlag, Berlin, pp. 251-282.

Hoppe, H.G. (1984) 'Attachment of bacteria: advantage or disavantage for survival in the aquatic environment', in K.C. Marshall (ed.), Microbial Adhesion and Aggegation, Springer-Verlag, Berlin, pp. 283-301.

Lowe, M. (1988) Ph.D. Thesis, University of Birmingham.

Lowe, M.J., Duddridge, J.E., Pritchard, A.M. and Bott, T.R. (1984) 'Biological-particulate fouling interactions: effects of suspended particles on biofilm development', Proceedings of the 1st UK National Heat Transfer Conference, Leeds, pp. 391-400.

Mansfield, G.H. (1983) Private Communication.

Novak, L. (1981) 'Control of the Rhine water fouling', in Somerscales and Knudsen (eds.), Fouling of Heat Transfer Equipment, Hemisphere Publish. Corp., N.Y., pp. 397-408.

Oliveira, D.R., Vieira, M.J., Melo L.F. and Pinheiro, M.M. (1990) 'Interaction between different fouling agents in water systems', Proceedings of the 9th International Heat Transfer Conference, Jerusalem, Hemisphere Publish. Corp., Vol. 5, pp. 103-108.

Olthof, M. and Oleszkiewicz, J. (1982) 'Anaerobic treatment of industrial wastewaters', in Process Technology and Flowsheets - Vol. II, McGraw-Hill, pp. 242-247.

Parker, J.G. and Roscow, J.A. (1981) 'The service performance of condenser tube materials in cooling waters containing appreciable suspended solids', Proceedings 8th Int. Congr. Metal Corrosion, Mainz, pp. 1285-1290.

Pinheiro, M.M. (1987) Ph. D. Thesis, University of Minho.

Pinheiro, M.M., Melo, L.F., Pinheiro, J.D. and Bott, T.R. (1988) 'A model for the interpretation of biofouling', Proceedings of the 2nd UK National Heat Transfer Conference, Glasgow, pp. 187-197.

Särner, E. (1986) 'Removal of particulate and dissolved organics in aerobic fixed-film biological processes', Journal of the Water Poll. Control Federation, 58, 165-172.

Stotzky, G. (1966) 'Influence of clay minerals on microorganisms - II. Effect. of various clay species, homionic clays and other particles on bacteria', Can. J. Microb., 12, 831-848.

Teixeira, F.J. (1988) 'Análise estatística das características físico-químicas dos efluentes fabris das unidades têxteis situadas nas áreas de Guimarães e Pevidém', Proceedings of 1º Encontro Nacional sobre Águas Industriais, ISEP, pp. 41-46 (in Portuguese).

Teixeira, F.J. and Silva, J.C. (1990) 'Evolução da qualidade das águas superficiais da bacia do Ave', Proceedings of 2º Encontro Nacional sobre Águas Industriais, ISEP, pp. 35-53 (in Portuguese).

Urbano, J.N. and Fernandes, A.M. (1988) 'Estação de tratamento de águas residuais da refinaria de Lisboa', Proceedings of 1º Encontro Nacional sobre águas industriais, ISEP, pp. 55-56 (in Portuguese).

Vieira, M.J., (1992) Ph. D. Thesis, University of Minho.

BIOFILMS AND CORROSION

A.H.L.CHAMBERLAIN
University of Surrey
School of Biological Sciences
Guildford GU2 5XH, United Kingdom

1. Introduction

The surface of metals can usually be considered as a shifting mosaic of anodes and cathodes, which continuously relocate as areas become more or less passivated compared to their surroundings. This usually results in the slow process of generalised attack over the whole surface or in the formation of overall passivating layers of metal corrosion products. The development of a biofilm at the surface will form a relatively stabilised matrix which may have numerous effects on the electrochemical processes but particularly in retaining a point source of chemical activity in a fixed position on or near the metal surface. It may be argued that this is also true in the case where a bacterium simply adheres to the metal surface, but there diffusion is relatively unimpeded, except over a very small contact area. In the case of the biofilm with its macromolecular gel structure diffusion can be restricted, and bearing in mind the heterogeneous distribution of the components, biological and abiotic, the development of differential concentration regions is of supreme importance.

The opposite question of whether corroding metals encourage biofilm formation by providing localised regions with more favourable physiochemical properties onto which microorganisms could adhere, is a point considered by Little et al.(1990).

Corrosion is essentially a metal attempting to return to its most stable form in the surrounding environment. The process by which this happens is the linkage of two reactions, the anodic reaction occurs as metal ions leave the metal surface with the stripped electrons passing through the metal to a second site, the cathode, where the electrons can be dissipated through reduction reactions. The material being reduced can vary depending upon the environment. The following reactions are most typical.

Neutral-alkaline, aerobic media:-

$$2H_2O + O_2 + 4e^- \rightarrow 4OH^- \qquad (1)$$

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 207–217.

Acidic, aerobic media:-

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \qquad (2)$$

Acidic, low oxygen-anaerobic media:-

$$2H^+ + 2e^- \rightarrow 2H \rightarrow H_2 \qquad (3)$$

However, under certain circumstances various other materials may be reduced e.g. in crevices on copper,

$$Cu^+ + e^- \rightarrow Cu^o \qquad (4)$$

leading to the deposition of nascent copper metal. Organic materials may also take part in cathodic reactions.

These corrosion reactions will take place if thermodynmically favoured in any moist medium. However, unless very stringent precautions are taken, surfaces in contact with non-toxic aqueous environments will inevitably develop a surface-attached microbial community. The types of organisms present, their overall numbers and relative populations will vary according to the environment with numerous factors relating to nutrients and physico-chemical parameters involved.

The process of biofilm formation on metallic surfaces is similar to that on any other surface, except that if corrosion can take place then corrosion products may become an integral part of the biofilm structure. This may modify the chemical and microbiological characteristics of the biofilm.

The result of corrosion is that the surface of the metal will become coated with one or usually more of an enormous range of corrosion products, which may include hydrated oxides, hydroxides and other basic salts. Some metals like titanium or high molybdenum stainless steels rapidly form a protective superficial coating which smothers the corrosion reactions and produces a passive state. However, most metals although forming layers of corrosion product and becoming stabilised may begin to corrode again rapidly if the localised chemical conditions change. It is by mediating this change of conditions that microorganisms can interact with metallic surfaces.

2. Biofilm formation

The development of a biofilm on a surface is a stepwise process. Initially adsorbed organic molecules bind irreversibly to the surface (see Chamberlain, this volume) to create a poorly defined interface. It is worth stressing that some corrosion products, such as iron hydroxides and hydrated oxides, may scavenge organics very effectively (Table 1), thus building up considerable reserves of carbon- and nitrogen-rich materials which may be available as nutrients to at least some of the members of the bacterial community.

Morel and Palenik (1989) indicated that trace metals and anions could also become bound onto oxide layers but that these processes were highly pH dependent. This may be significant as one of the major features of active biofilm presence on a surface is its ability to modulate

the pH close to the surface, often in a rather "patchy" way. It is also possible that the organic molecules actively compete for at least some of the oxide metal binding sites and also physically block others so the final position is almost impossible to predict.

TABLE 1. Atomic percentages of carbon, oxygen and nitrogen following adsorption of dissolved marine organics onto mild steel or rust deposits.

Material and contact time (hours)	Organic Concentration mgl^{-1}	Relative atomic percentage		
		Carbon	Oxygen	Nitrogen
Metal, 1	1	28.5	36.7	trace
	10	29.2	41.2	trace
	100	29.8	41.1	trace
Metal, 3	1	27.7	49.9	0.5
	10	31.2	48.5	0.3
	100	30.8	49.0	trace
Rust,3	1	36.3	23.7	1.0
	10	39.5	31.5	0.7
	100	35.0	26.3	1.4

The composition of the biofilm itself is again highly dependent upon the environmental conditions. Initial attachment of individual bacteria will give rise to a very disjunct community, and the development of this will be a feature of the mass transfer conditions, largely controlled by the number of cells in suspension, the flow velocity and turbulence of the medium.

The biological components of biofilms are not restricted to bacteria but may also include Fungi, Protozoa and of particular importance in illuminated environments, Algae and Cyanobacteria

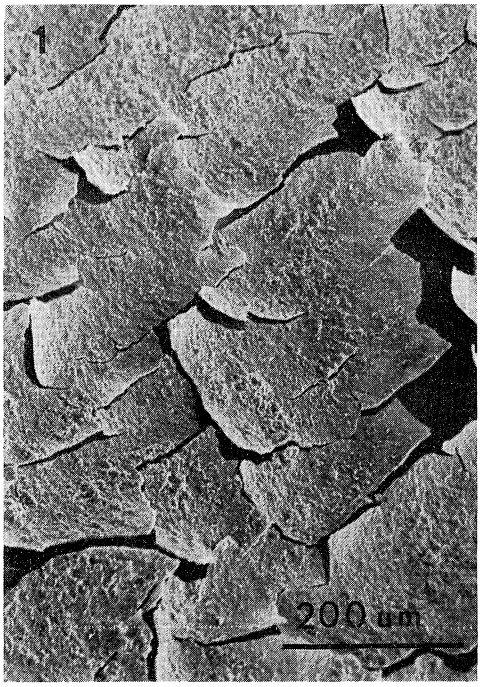
Once firm attachment has occurred cells may grow on the surface at the expense of bulk or surface-bound nutrients, leading to cell division which ultimately creates a mosaic of micro-colonies across the surface. Under certain conditions these may become confluent to achieve a complete surface layer, but the colonies may remain distinct for a long time. Fletcher and Floodgate (1973) indicated that adhesion of bacteria is frequently followed by the secretion of extracellular polymeric substances (see also Cooksey, this volume). These materials are of the utmost importance in biofilm formation and represent the so-called biofilm matrix which acts as the adhesive holding the entire structure together. The nature of this matrix can be very variable in chemical composition and physical properties, and both of these aspects may have a profound effect on the biofilms' influence on the corrosion process. Some algae too may contribute large volumes of polysaccharide extracellular mucilage. The diatoms are especially important, where the polymers are mainly acidic, due to uronic acids or half - ester sulphate groups (Daniel et al.,1980)

The biofilm is visualised as a thin film of highly hydrated polymeric material containing some 95%+ of water and showing visco-elastic properties. The chemical nature of the matrix is frequently complex, but is predominantly polysaccharide. The type and number of reactive

groups, such as uronic carboxyl, side-chain carboxyls(e.g.pyruvate carboxyls in xanthans), amino groups and others such as phosphate, which may have a considerable role in binding reactions, depend upon the species present and the nutrient levels. These matrix materials are produced by the bacteria within the surface community and each species may produce a polymer which differs in basic composition or stereochemistry. This means that across a biofilm a mosaic of matrix polymers may exist both in the horizontal and verical dimensions.

The microbial cells themselves are also an important component of the biofilm as their metabolic activities not only supply the matrix polymers but also other low and high molecular weight compounds, and act as a point sink for nutrients and oxygen if heterotrophic. Photoautotrophs, such as algae and cyanobacteria, will produce oxygen when illuminated, but will deplete CO_2 or HCO_3^- which can have major effects on pH and buffering capacity (see later).

In addition to the microorganisms and their extracellular secretions, biofilms may also contain numerous other components including particulate organic materials such as humic materials (Fig.1), inorganic mineral particles from the medium, such as clay minerals (Fig.2), together with inorganic corrosion products derived from metallic surfaces in the system (Fig.3). All of these components will be active adsorption sites for organic and inorganic species and hence the composite biofilm can be seen as a highly complex, highly reactive and interactive layer applied intimately to the metallic surface.



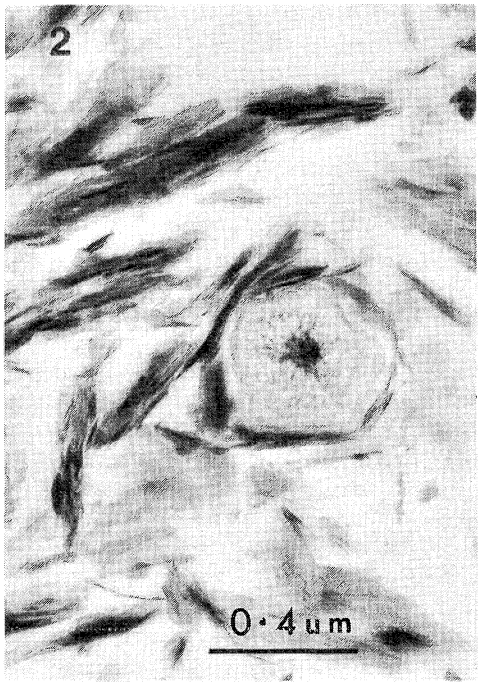


Figure 1. An SEM of a humic acid rich surface film on copper pipe.
Figure 2. A transmission electron micrograph of a bacterium surrounded by clay mineral particles in a marine biofilm.

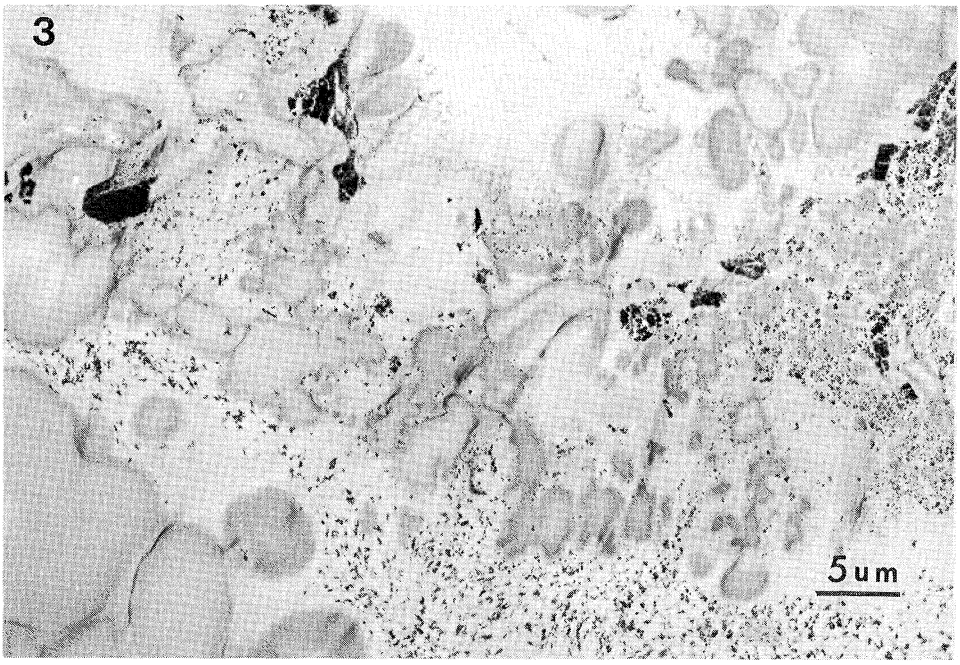


Figure 3. Lipid-rich film on mild steel with associated finely particulate corrosion products.

The question to be considered now is what roles does this family of structures play in metal corrosion? This may be divided into a number of facets:-

a) Physical effects
- diffusion barrier
- reduction of turbulence & surface flow
- reduction of heat transfer where applicable

b) Chemical effects - these can be considered as acting on the anodic reaction, the cathodic reaction, or influencing both.

i) Anodic reaction - prevention of anodic polarisation through metal ion complexation and/or binding.

ii) Cathodic reaction - modulation of reducible species.
- supply of reducible species

iii) Dissolution of protective oxide/hydroxide layers.

iv) Selectivity of ion permeability.

v) Effect upon pH.

However, the most important aspect of all the diffusion and chemical effects is the creation of differential concentration cells producing galvanic corrosion cells.

3. Oxygen diffusion

It is necessary to point out that rate of O_2 diffusion through the extremely hydrated polymeric matrix itself is very unlikely to be significantly different from that of water. It is only in the presence of bacteria carrying out aerobic respiration that O_2 diffusion will be limited and as described above, can lead to complete anaerobiosis at the biofilm/metal interface.

This opens up a further question, not of oxygen consumption but its generation at metallic surfaces, particularly if they are illuminated. Some preliminary ideas on photochemical events at a biofilmed, metallic surface have been put forward by Morel and Palenik (1989), but the development of biofilms containing organisms carrying out oxygenic photosynthesis will result in the delivery of pure oxygen directly onto the metal surface, stimulating the cathodic reaction. This was proposed as a key feature of the formation of thick films of the diatom Amphora on 90:10 copper-nickel in marine environments and the subsequent sloughing of the entire corrosion product/biofilm complex (Chamberlain and Garner,1988).

Schiffrin and de Sanchez (1985), using well defined rotating disc electrode techniques, were able to show that on copper and copper alloy surfaces, biofilm effects on corrosion could be of two types, either sulphide generation (via SRBs or aerobic proteolysis), or oxygen diffusion limitation. In the latter, current densities were more than doubled in seawater saturated with oxygen. Elevated oxygen levels can also raise the metabolic rate of the heterotrophic community, leading to enhanced secretion of metabolites, some of which may further influence the corrosion reactions.

Regions of biofilmed metal which have been exposed to sulphide as a result of anaerobic SRB activity and have incorporated metal sulphide(s) into the surface oxide layers often show very high corrosion rates when exposed to oxygen (Chamberlain et al.,1988b).

There are some bacteria, which although anaerobic are able to carry out a form of photosynthesis which generates not oxygen but sulphur. This is an extremely aggressive element to many metals and has been shown to cause considerable damage (Cragnolino and Tuovinen, 1984).

4. Effects of pH

The pH of the electrolyte has a profound effect on the chemistry of the cathodic reaction and its product(s) and also on the stability of the protective layers. Thus, the dissolution of an oxide layer will enable the anodic process to restart. This can also be a major factor where cathodic protection has been applied as a means of preventing corrosion. The effectiveness of the cathodic protection depends at least in part on the precipitation of calcareous scales due to the high pH produced by the forced generation of OH^- ions. If this calcareous scale is dissolved by acidic secretions, then further continued corrosion will occur. A recent computer model, developed by Dexter and Lin (1992), and supported by microelectrode measurements, suggested that calculated values of pH at applied current densities of less than $20\mu Acm^{-2}$ could be seriously in error in the presence of actively metabolising organisms. Calculation showed that where oxygen reduction on steel in seawater was the sole cathodic reaction, the maximum surface pH obtainable is 9.9. However, if H_2 evolution occurred higher pHs were possible. Simple impressed current experiments using electrodes buried in agar containing BDH Universal Indicator (van Woerkom and Chamberlain, unpublished), suggest that values close to pH 11 are

demonstrable.

Lewandowski et al.(1989) also utilised pH microelectrodes to investigate biofilms on metallic surfaces, again under cathodic polarisation, and indeed showed that beyond the point of H_2 evolution, the pH continues to rise due to the following reaction:-

$$2H_2O + 2e^- \rightarrow H_2 + 2OH^- \qquad (5)$$

They were also able to show that the medium, 3.5% sodium chloride solution or artificial seawater, had a significant effect on the pH obtained, because the bicarbonate in seawater buffered the system below pH 10, whilst the NaCl rose to pH 10.5. The presence of microorganisms can significantly effect the buffering capacity of the medium in close contact with the metal surface. They also caused a decrease in the measured current density by competing for the dissolved oxygen. This may be of considerable significance in cathodically protected systems. If the O_2 levels are scavenged to very low values, then there will not be enough OH^- to generate the calcareous films.

The oxygen consumption by the microorganisms in the film was sufficient to reduce the O_2 levels completely, giving anaerobic conditions at the base of the film.

Such a process is also highly likely to occur in many situations where deposit attack takes place. Although there are undoubtedly some instances where high levels of deposit in slow moving water act independently, staining techniques indicate biopolymer matrix is frequently present (Chamberlain and Nuttall, unpublished).

The formation of biofilms on surfaces is a completely natural process and can even occur on what might be considered toxic materials. For instance, copper and its alloys have been considered "naturally antifouling" because of the slow release of copper (II) ions. However, although this mechanism may be effective on initial immersion, and even last for several weeks, the surfaces do eventually develop biofilms of organisms which are "selected" by the surface due to their copper tolerance. Similar results have been obtained with silver. Once established, these biofilms may then be an agency for corrosion to occur.

The production of extracellular polymers appears to play a key role in this protection from toxic metals as described for Klebsiella aerogenes exposed to copper and cadmium ions (Bitton and Freihofer, 1978). Corpe (1975) showed a similar effect of extracellular polymers produced by marine bacteria exposed to copper. Angell and Chamberlain (1990) also found additional quantities of polysaccharide polymers were produced by Pseudomonas paucimobilis (now Sphingomonas paucimobilis) in the presence of copper.

There is ample evidence, particularly from the results of Geesey and his co-workers (Geesey and Bremer, 1990), that matrix polymer alone may be capable of at least initiating corrosion in the case of copper. It is of some interest that the dissolution of a thin copper film from the surface of an FTIR - Internal Reflection Element (IRE) by a bacterial isolate appeared to be correlated with flow conditions (Bremer and Geesey, 1991a). During a 330h flow regime no deterioration of the copper occurred, but shortly after flow was stopped, the calculated corrosion rate rose ten-fold. Bremer and Geesey (1991b) were also able to show an increase in the quantity of exopolysaccharide on the copper surface concomitant with the elevated corrosion, and could show that under static conditions, copper dissolution was equally rapid.

However, it is important to recognise that biofilm matrix is an extremely complex mixture of materials and will contain a variety of functional groups capable of binding metal ions. Ford, Maki and Mitchell (1987) showed that on further purification of a polymeric matrix isolated

from Deleya marina grown in suspension, the loss of metal binding capacity was mirrored by a loss of protein. Bremer and Geesey (1992) have also noted this feature, and certainly it was apparent using the staining procedures developed by Chamberlain et al. (1988) that proteins are usually present in natural and many artificial biofilm matrices.

Detailed examination of these various publications, however, does suggest that considerable emphasis has been placed on binding studies and analyses of polymers produced by cells in batch, suspension cultures. More recent work by Angell and Chamberlain (1991) has shown that polymers produced by aerobic bacterial cells on surfaces may be quite different from those in suspension culture. A similar situation was demonstrated by Beech et al.(1991) for the anaerobe Desulfovibrio desulfuricans. Thus, although the detailed metal ion binding work is of interest, it may not be of direct relevance to the corrosion scenario. Nevertheless, there may be good arguments to consider what corrosion activities are possessed by organisms growing in suspension culture,as this may often be the preceding state before the movement of cells onto a surface. The topic of this communication, however, is to consider the biofilm situation.

It is perhaps worth considering if all metals can become substrata for biofilm development. There are certainly some metals which only become colonised very slowly in nature. Barrett and Chamberlain (unpublished) found that silver mesh remained essentially biofilm-free in a river system over a period of several months. Silver coupons also remained "clean" for several months in a marine harbour environment, whilst gold was rapidly colonised (Chamberlain, unpublished). The other main series of materials which show some resistance to film formation is copper and its alloys, especially the copper-nickels; Efird (1976) also suggested zinc in seawater. Binding of the toxic metal ions by the biofilm matrix allows subsequent colonisation of a surface by organisms with little or no resistance.

It is probably worth re-iterating an observation by Dempsey (1981) that the leaching of toxic ions may actually encourage biofilm formation by metal tolerant bacteria, because competitors and predators are predominantly repelled or killed.

5. Corrosion detection methods

Recently several review papers have considered electrochemical techniques for detecting and attempting to interpret the mechanisms of microbial corrosion (Dexter et al.,1989; Little et al., 1991; Mansfeld and Little, 1991). The overriding problem where microorganisms are involved lies in the prevalence of pitting or highly localised corrosion, so that many techniques which are standard in the corrosion field are unable to distinguish these types of attack. Simple corrosion potential, redox potential or polarisation resistance measurements alone cannot usually yield sufficient information for successful interpretation of the role microorganisms might be playing. However some techniques, such as electrochemical impedence spectroscopy, show considerable promise (Franklin et al., 1991a) although the interpretation of the derived spectra is often difficult. Conventional polarisation sweeps, used in conjunction with supporting techniques like scanning electron microscopy and energy dispersive x-ray analysis, are still a valuable source of information about possible electrode reactions but care must be taken to limit the range of the sweeps or major damage to the surface film can give totally distorted impressions (Dowling et al., 1988).

A recent further report by Franklin et al. (1991b) has introduced a useful, non-destructive technique which shows considerable promise for the investigation of the rather localised events

associated with biofilm/MIC; the scanning vibrating electrode. This instrument was used to map the current density over a working electrode surface and, in combination with open circuit potential and polarisation resistance (Rp) data, showed that in sterile medium, or spent growth medium from which all organisms had been removed by centrifugation, the normal initiation of pits was quickly followed by repassivation. Where the experiments were repeated in the presence of a Pseudomonas the open circuit potential fell below -600mV(SCE), whereas in sterile medium it rose above -200mV(SCE), and initiated pitting continued to propagate. The suggestion was advanced that the biofilm created by the pseudomonad with its copious matrix polymer could produce areas coated by 'membranes' that inhibit the diffusion of aggressive ions out of the pits, or passivating ions, such as phosphate, inwards. A similar concept of 'membrane-like' properties for microbial biofilm involved in corrosion has been advanced by Wagner et al.(1992) in the case of copper pipework carrying certain types of freshwater.

6. Conclusions

Despite the difficulties in proving conclusively that a corrosion event has been caused exclusively through microbial activity, there is now a general acceptance that it can and does occur on a wide range of metals and their alloys (Dowling et al.,1991). Many different mechanisms have been proposed, some as a result of extrapolating from the known metabolic pathways of various groups of organisms, others as a result of careful examination of corrosion products and the subsequent controlled modelling of a system under laboratory conditions. It is important that such laboratory-based test systems should attempt to reproduce as accurately as possible the operating parameters and conditions where the corrosion was originally experienced. This is especially important in the case of the fluid medium. Castle et al.(1984) and Castle et al.(1988) showed that the adsorption of dissolved organics onto copper-nickel played a significant role in the cathodic reaction at low oxygen tensions. Also, rather different corrosion product minerals may form in a 'living' seawater compared to an artificial, synthetic medium.

The preceding pages show that, in most environments which experience temperatures above freezing and below about 70°C, a metal exposed to an aqueous medium containing an inoculum of microorganisms with sufficient nutrients to grow, will develop a surface biofilm and in many circumstances undergo some form of microbially associated corrosion. In most cases, this will be due to the establishment of some heterogeneity across the metal surface, leading to some form of pitting.

7. References

Angell, P. and Chamberlain, A.H.L. (1990) 'The role of extracellular products in copper colonisation', International Biodeterioration 27, 135-143.

Beech, I.B., Gaylarde, C.C., Smith,J.J. and Geesey, G.G. (1991) 'Polysaccharides in biofilms and in free EPS of D.desulfuricans in the presence of mild and stainless steel and their role in corrosion', in N.J.Dowling, M.W.Mittelman and J.C.Danko (eds.),Microbially Influenced Corrosion and Biodeterioration, MIC consortium, Knoxville, pp.2.53-2.63.

Bitton, G. and Freihofer, V. (1978) 'Influence of extracellular polysaccharides on the toxicity of copper and cadmium toward Klebsiella aerogenes ', Microbial Ecology 4, 119-125.

Bremer, P.J. and Geesey, G.G. (1991a) 'An evaluation of biofilm development utilising non-destructive attenuated total reflectance Fourier transform infrared spectroscopy, Biofouling, 3, 89-100.

Bremer, P.J. and Geesey, G.G. (1991b) 'Laboratory-based model of microbiologically induced corrosion of copper', Applied and Environmental Microbiology, 57, 1956-1962.

Bremer, P.J. and Geesey, G.G. (1992) 'Properties of the exopolymer of a bacterium that causes pitting corrosion of copper', Dev.Indust. Microbiol. (in press)

Castle, J.E., Chamberlain,A.H.L., Garner, B., Parvizi, S. and Aladjem, A.(1988) 'The use of synthetic or natural seawater in studies of the corrosion of copper alloys', in P.E.Francis and T.S.Lee (eds.), The Use of Synthetic Environments for Corrosion Testing, ASTM, Philadelphia, pp. 174-189.

Castle, J.E., Parvizi, M.S. and Chamberlain, A.H.L. (1984) 'Interaction of marine biofouling and corrosion on copper based alloys', in Microbial Corrosion, The Metals Society, London, pp. 36-45.

Chamberlain, A.H.L. and Garner, B.J. (1988) 'Microbial fouling and corrosion of 90/10 copper-nickel alloys', in C.A.C.Sequeira and A.K.Tiller (eds.), Microbial Corrosion 1, Elsevier Applied Science, London, pp. 431-439.

Chamberlain, A.H.L., Angell, P.J. and Campbell, H.S. (1988) 'Staining procedures for characterising biofilms in corrosion investigations', Br. Corros. J., 23, 197-198.

Chamberlain, A.H.L. and Nuttall, J. (1990) 'Demonstration of biopolymers associated with materials causing deposit attack', unpublished.

Chamberlain, A.H.L., Simmonds, S.E. and Garner, B.J. (1988b) ',Marine "copper-tolerant" sulphate reducing bacteria and their effects on 90/10 copper-nickel(CA706)', International Biodeterioration, 24, 213-219.

Cooksey, K.E.(1992) 'Extracellular polymers in biofilms', in Melo et al.(eds.), Biofilms: Science and Technology, Kluwer Academic Publishers, Dordrecht, The Netherlands, this volume.

Corpe, W.A. (1972) 'Microfouling: the role of primary film forming marine bacteria ', Proceedings of the 3rd International Congress on Marine Corrosion and Fouling, pp. 598-606.

Cragnolino, G. and Tuovinen, O.H. (1984) 'The role of sulphate- reducing and sulphur-oxidizing bacteria in the localised corrosion of iron-base alloys - a review', International Biodeterioration, 20, 9-26.

Daniel, G.F., Chamberlain, A.H.L. and Jones, E.B.G. (1980) 'Ultrastructural observations on the marine fouling diatom Amphora ', Helgoländer Meeresunters., 34, 123-149.

Dempsey, M.J. (1981) 'Colonisation of antifouling paints by marine bacteria', Botanica Marina, 24, 185-191.

Dexter, S.C. and Lin, S.H. (1992) 'Calculation of seawater pH at polarized metal surfaces in the presence of surface films', Corrosion, NACE 48, 50-60.

Dexter, S.C., Siebert, O.W., Duquette, D.J. and Videla, H.A. (1989)' Use and limitations of electrochemical techniques for investigating microbiological corrosion',Corrosion/89, Paper No. 616, pp. 616/1-22.

Dowling, N.J., Mittelman,M.W. and Danko, J.C. (1991) (eds.) Microbially Influenced Corrosion and Biodeterioration, University of Tennessee, Knoxville.

Efird, K.D. (1976) 'The inter-relation of corrosion and fouling for metals in sea water', Materials Performance, 15,16-25.

Fletcher, M. and Floodgate, G.D. (1973) 'An electron microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces',J. Gen. Microbiol. 74, 325-334.

Ford, T.E., Maki, J.S. and Mitchell, R. (1987) 'The role of metal-binding bacterial exopolymers in corrosion processes', Corrosion/87, Paper No. 380, pp. 380/1-7.

Franklin, M.J., Nivens,D.E., Guckert, J.B. and White, D.C. (1991a) 'Effect of electrochemical impedance spectroscopy on microbial biofilm cell numbers, viability, and activity', Corrosion-NACE, 47, 519-522.

Franklin, M.J., White, D.C. and Isaacs, H.S. (1991b) 'Pitting corrosion by bacteria on carbon steel, determined by the scanning vibrating electrode technique', Corrosion Science, 32, 945-952.

Lewandowsky, Z., Lee,W.C., Characklis, W.G. and Little, B.(1989) 'Dissolved oxygen and pH microelectrode measurements at water-immersed metal surfaces ', Corrosion NACE 45, 92-98.

Little, B., Wagner,P.A., Characklis, W.G. and Lee, W. (1990) 'Microbial corrosion', in W.G.Characklis and K.C.Marshall (eds.), Biofilms, John Wiley & Sons, New York, pp. 635-670.

Little, B., Wagner, P. and Mansfeld, F. (1991) 'Microbiologically influenced corrosion of metals and alloys' , International Materials Reviews, 36, 253-272.

Mansfeld, F. and Little, B. (1991) 'A technical review of electrochemical techniques applied to microbiologically influenced corrosion ',Corrosion Science, 32, 247-272.

Morel, F.M.M. and Palenik, B. (1989) 'The aquatic chemistry of trace metals in biofilms ', in W.G.Characklis and P.A.Wilderer (eds.), Structure and Function of Biofilms, John Wiley & Sons Ltd, Chichester, pp. 289-300.

Schiffrin, D.J. and De Sanchez, S.R. (1985) 'The effect of pollutants and bacterial microfouling on the corrosion of copper base alloys in seawater', Corrosion NACE 41, 31-38.

Chapter 4

PROCESS ANALYSIS AND BIOFILM MODELING

BIOFILM ACCUMULATION & ACTIVITY: A PROCESS ANALYSIS APPROACH

JAMES D. BRYERS
THE CENTER FOR
BIOCHEMICAL ENGINEERING
DUKE UNIVERSITY
DURHAM, NORTH CAROLINA
27706 USA

WILLIAM G. CHARACKLIS
THE CENTER FOR INTERFACIAL
MICROBIAL PROCESS ENGINEERING
MONTANA STATE UNIVERSITY
BOZEMAN, MONTANA
59717 USA

1. Introduction

"What is the good of that," said Rabbit.
A.A. Milne, The House at Pooh Corner.

Process Analysis refers to the application of systematic methods to recognize, define and clarify problems and to develop methodologies for their solution. Biofilm formation and persistence in both natural and engineered systems is governed by a collage of complex physical, chemical, and biological processes; each process dependent on a unique set of system parameters. Process analysis applied to biofilm formation provides an integrated approach which incorporates microbial physiology, reaction engineering, and transport phenomena to understand, control, and exploit biofilm processes. Application of process analysis allows one to *(a)* interpret the operation of an existing biofilm system, *(b)* design new biofilm reactor systems, and *(c)* understand the complexities of natural biofilm systems. It is increasingly apparent that research into biofilm processes which does not comprise microbial, chemical, and fundamental engineering aspects is incomplete.

Here, we will present the concepts of *process analysis* and the *rate concept* approach to mathematically describing complex reaction systems as exemplified by the formation and persistence of a biofilm. The basis of process analysis lies in the fundamental conservation equations of mass, energy, and momentum. Application of process analysis and the rate concept provides a systematic protocol with which to either interpret an existing reaction system or to design and operate a new system. First, we will define various model types and their relative utility with special attention paid to both structured and unstructured model concepts. Second, those processes contributing to the formation and persistence of a biofilm will be enumerated and the individual rate expressions for these fundamental processes discussed. Errors associated with ignoring the involvement of these individual rate processes in cell adhesion and biofilm formation are also described.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 221–237.

2. Process Analysis, the Rate Concept, and Mathematical Modeling

It is one of the maxims of civil law, that definitions are hazardous.
Samuel Johnson

Process analysis is simply a method of mathematical describing a complex phenomena as the net result of a number of individual fundamental processes. The *rate concept* more specifically assumes that changes in the state of a system with time can be systematically treated as the summation of the rates of the individual processes acting on the system. As implied, some degree of modeling of the biological system is posed, predictions of the model compared to observation, and the model verified or refuted. Thus, modeling must be, by nature, an evolutionary process in order to provide insight about a system.

"...as we advance into the terra incognita of tomorrow, it is better to have a general and incomplete map, subject to revision and correction, than to have no map at all.
Alvin Toffler, Power Shift, p. xxi

Modeling is basically the scientific process of forming and testing a hypothesis. A model need not be mathematical nor need it exactly replicate reality. A road map is a model of a section of the Earth that allows one to successfully navigate from point A to B without representing every house or structure that actually exists. Models need not (and can not) simulate all details of a system to be of use. Models are simply our inherent tendency to explain unknown phenomena based on perceived principles. A good portion of basic science is predicated on proving or refuting such hypotheses. Mathematical models do provide a rigid structure with which to formulate concepts regarding a complex system. Mathematical models provide a means of verifying the goodness of a model by way of many statistical criteria. Important variables and system parameters can be easily identified and, through the use of dimensionless groups of such variables, mathematical models can expedite experimentation. And should the mathematical model prove successful in correlating observable data from a system at one set of conditions, then one has the ability to extrapolate; predicting the responses of the system under a variety of different conditions.

Though this be madness, yet there is method in't.
Shakespeare, Hamlet, Act II, Sc. 2

Three basic types of models are: *(1)* transport or continuum models, *(2)* population models, and *(3)* empirical or statistical models. Residence time distributions and synchronous growth are examples of population models while statistical regression of data sets is an example of empirical models. Transport models, the type considered here, essentially account for the changes of either mass, energy, or momentum in a continuum or defined control volume.

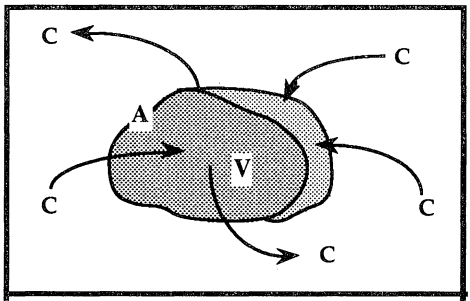


Figure 1. Continuum Approach to the Conservation of Mass

Consider the control volume illustrated in Figure 1. The control volume can exchange mass, energy, and momentum with its surroundings as indicated by the arrows. The conservation equation for mass equates the rate of accumulation within the control volume to the net rate of input into the control volume plus the summation of all process rates producing or consuming mass in the volume. A verbal form of the conservation equation for the mass of a component C can be expressed as follows:

Net rate of accumulation of C in control volume = **+ rate of transport of C into the volume across area A**
- rate of transport of C out of the volume across area A
+ Σ (all transformation processes generating or consuming C within the volume V)

The net accumulation rate and the rates of transport into and out of the volume are termed *rates of change* or *system rates* in that they represent the observed change in the measured component. System rates are extensive quantities and are by definition system-specific and can not be correlated to fundamental parameters (*e.g.*, temperature, velocity) describing the system. Unfortunately, all too often many try.

Transport rates comprise *bulk transport* (movement of component due to the flow of the bulk liquid), *interfacial or interphase transport* (transport across the interface between two phases), and *intraphase transport* (transport within one phase due to a gradient - *e.g.*, diffusion). One common criterion regarding a transport rate is that changes in a component's concentration (in the case of mass transport) are not due to a molecular change in the component.

Conversely, process rates describe transformations that occur due to either chemical, biochemical, or biological reactions that either produce or consume the component. Process rates are fundamental intensive quantities in that they can be correlated to system parameters such as temperature, pressure, concentration, and velocity. Process rates and their correlation to such fundamental parameters are independent of the system in which they occur.

Process or transformation rates comprise two fundamental concepts: *stoichiometry* and *reaction rate kinetics*. Stoichiometry provides the relative basic molar quantities of the various reactants and products within a balanced reaction. Stoichiometry for a given set of components comprising a reaction establish the thermodynamics of the system. For example, consider the chemical oxidation of glucose,

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad \mathbf{r} \qquad (1)$$

which states that 1 mole of glucose reacts with 6 moles of oxygen to form 6 moles of carbon dioxide and water. Mathematically, a reaction stoichiometry for N number of reaction components M can be expressed as,

$$\sum_{i=1}^{N} \mathbf{a}_i \mathbf{M}_i = 0 \tag{2}$$

where a_i is the stoichoimetric coefficient of the i-th component and M_i is component i. The convention is that stoichiometric coefficients, $\mathbf{a}_i$, for reactants have negative values while products have positive coefficients - *e.g.*, in Eqn. (1), the coefficients for glucose, oxygen, carbon dioxide, and water are -1, -6, +6, and +6, respectively. The term **r** in Eqn. (1) is the rate of reaction and has units [moles of reaction/volume-time]. Stoichiometry allows one to relate the rates of appearance of all components to each other as follows,

$$\mathbf{r} = \frac{\mathbf{r}_1}{\mathbf{a}_1} = \frac{\mathbf{r}_2}{\mathbf{a}_2} = \ldots = \frac{\mathbf{r}_i}{\mathbf{a}_i} \tag{3}$$

where r_1 = the rate of appearance of component 1, and so on.

The other portion of the process rate is the reaction rate itself. While stoichiometry relates to the molar ratios of components in a transformation, kinetics refers to how fast the transformation occurs. If a reaction rate is *homogeneous*, it is said to occur uniformly throughout the reaction volume V and is expressed as the number of moles reacted per unit volume per time. *Heterogeneous* reactions occur at the interface between two phases and do not occur uniformly throughout the reaction volume; heterogeneous reaction rates are more commonly expressed on a per unit surface area or per unit weight of reacting surface. Rate expressions are mathematical functions that relate the reaction rate to basic fundamental parameters, - *e.g.*,

$$\mathbf{r} = f(\text{temperature, concentration, etc.})$$

Typically, chemical reactions are described assuming this function can be written as the product of a number of separate functions,

$$\mathbf{r} = f_1(\text{temperature}) f_2(M_1, M_2, \ldots M_i)$$

For example, in chemical kinetics, the most common approach is the power-law expression where,

$$\mathbf{r} = k\,(M_1)^w\,(M_2)^x \ldots (M_i)^z \tag{4}$$

and k is a function of temperature only. The exponents in Eqn. (4) are referred to as the *orders* of the components in the reaction where in this example,

$$\mathbf{r} = k\,C^1 D^{0.5} E^{-2} \tag{5}$$

the reaction rate is first order in component C and half order in component D. The reaction rate **r** varies with the concentration of E to the -2 power.

It is critical to further discussions that the distinction between a process rate and a system rate be clear. Consider in Figure 2, a mass balance on component C that reacts in a reaction vol-

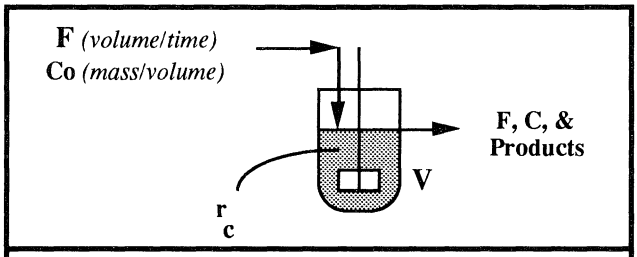


Figure 2. Mass Balance for C over an Ideal Continuously Fed Stirred Tank Reactor

ume V receiving a continuous supply of reactant C at an inlet concentration C_0. A homogeneous reaction $a_C C \rightarrow a_D D$ occurs in the reactor and its rate expression can be assumed as, $r_C = a_C k C^x$. It is assumed that mixing in the volume is sufficient to eliminate any concentration gradients. A mass balance on the reactor, illustrated in Figure 2, can be stated verbally as follows. . .

The Time Rate of Change of the Mass of Component C	=	**Net Transport Rate of Component C Into the Reactor**	+	**All Reaction Rates Producing or Component C**

or mathematically,

$$VdC/dt = F(C_0 - C) + r_C V \qquad (6)$$

Note that the first term in Eqn. (6) describes the rate of change of C in the system. The second term describes the rate component C is transported into and from the system. Both terms are system rates - *i.e.*, observable rates. The third term in the equation pertains to those process rates which describe how the reaction rate r changes as a function of the concentration C; if this rate expression truly represents the chemical or biological reaction, then it is valid irrespective of the system in which the reaction occurs. If the reaction rate, r_C, were a heterogeneous reaction rate written on a *per unit surface area* basis then the last term of Eqn. (6) would read instead, $r_C A$.

In the remaining sections of this paper we will present the various process rates that contribute to the net rate of change of biofilm mass or active cell concentration on a substratum. Those processes governing biofilm formation and persistence will be reviewed and rate expressions for each process discussed. Differences between structured and unstructured models of biofilm formation are presented detailed elsewhere in this volume by Bryers in the chapter on mixed population biofilms.

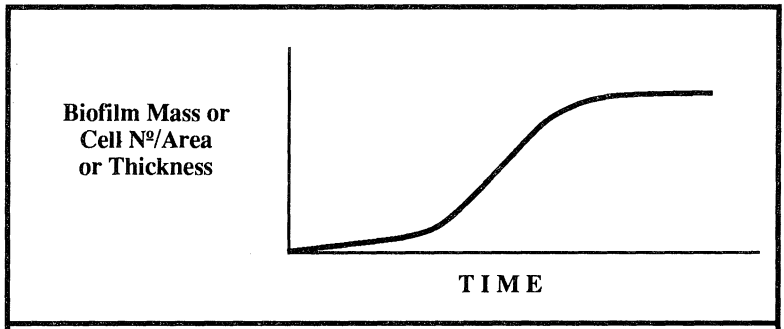


Figure 3. Biofilm net accumulation as a function of time.

3. Net Biofilm Accumulation

Figure 3 illustrates, irrespective of the analytical measure, the observed surface concentration of biofilm at a substratum as a function of time. Unfortunately, this type of data allows an estimate of only the rate of biofilm accumulating at the surface (*i.e.*, the slope of the curve); such a rate is a *system rate* or *rate of change*. Unfortunately, all too often, rates from such data are used to predict the stoichiometry and kinetics of the fundamental processes that contribute to the observed accumulation. In this section, we will describe the individual processes that collectively affect biofilm formation and provide the most current rate expression that most accurately describes that process.

Biofilm accumulation (Figure 4) is considered the net result of the

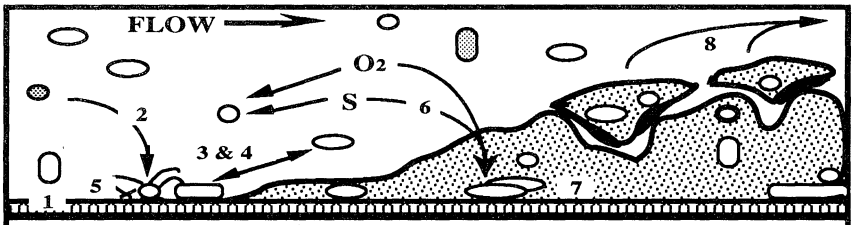


Figure 4. Fundamental processes governing biofilm formation. Definition of individual processes are given in the text below.

following physical, chemical and biological processes:

1. Biasing or pre-conditioning of the substratum either by macromolecules present in the bulk liquid or intentionally, as in the case of pre-coating endoprosthetic biomaterials with adhesion molecules (*e.g.*, fibronectin, vitronectin, fibrinogen, von Willebrand's factor).

2. Transport of planktonic cells from the bulk liquid to the substratum.

3. Reversible adsorption of cells at the substratum for a finite time.

4. Desorption is the release of reversibly adsorbed cells due fluid shear forces.

5. Irreversible adsorption is where cells remain permanent adsorbed to the surface.

6. Substrate metabolism by the attached cells.

7. Cell growth, replication, and extracellular polymer production.

8. Detachment of biofilm material can be either continuous, as in the case of erosion brought on by shear forces, or random, as in the case of biofilm sloughing.

If all of these processes occur in series, then the slowest rate process in the sequence will exert the greatest influence and limit the overall accumulation of biofilm. This slowest process is the *rate-limiting step* in that the overall accumulation of biofilm can go no faster than this slowest rate. Identifying the rate limiting step in a collage of processes such as biofilm formation is critical to data interpretation and potential scale-up. For example, if the goal is to derive the kinetics of cell adhesion but the system is *rate-limited* by the step of cell transport to the substratum, then any resultant kinetic rate expression for cell adhesion would be erroneous. Process analysis methods provide the protocol to analyze complex phenomenon as the summation of individual rates.

3.1 DEPOSITION PROCESSES

Process rates 1-5 are often grouped in mathematical models as single process termed "deposition". However, correlating "deposition rates" to system parameters, such as velocity or planktonic cell concentrations, is not sound *process analysis*.

3.1.1. *Pre-conditioning of the Substratum.* In the case of intentional pre-treatment of a surface, (*i.e.*, adhesion molecules for biomaterials), there is no rate to considered; the substratum essentially enters the system with a biased surface chemistry. However, in cases where a "clean" substratum material is exposed to an aqueous environment, transport of dissolved organic molecules or macromolecules in laminar flow is basically by molecular diffusion; in turbulent flow, transport must also consider eddy diffusion effects. Once at the surface, adsorption of macromolecules occurs almost instantaneously, immediately changing the surface chemistry of the exposed material. Loeb & Neihof (1975) and Depalma *et al.* (1979) have measured adsorption rates of organic molecules to various solid substrata in seawater and Bryers (1980) has observed analogous adsorption rates in a freshwater laboratory system. The net rate of adsorption in these studies can be described as...

$$r_{adsorption} = k_a S_i[1 - S_i/k_s] \qquad (7)$$

where $r_{adsorption}$ = the rate of net adsorption ($ML^{-2}t^{-1}$); k_a = adsorption rate constant, (Lt^{-1}); k_s = surface saturation coefficient, (ML^{-2}); and S_i = areal concentration of adsorbed species, (ML^{-2}). While experiments indicate the maximum amount of adsorbed material may not exceed 0.10 μm in thickness, the surface properties resulting from adsorption of an organic film can significantly bias subsequent microbial events.

3.1.2. *Cellular Adsorption and Desorption.* Observations have indicated that cells can adsorb to a surface for a period of time and then may desorb from the substratum, returning to the bulk liquid. If cells spend sufficient time at the surface, they can become permanently bound, perhaps due to extracellular polymer production, and can only be removed by rather aggressive physical or chemical means. One can conceive of this overall process much like two reaction steps in series, with the reversible adsorption process first followed by an irreversible step pertaining to permanent adsorption,

$$\underset{\text{Suspended Cells}}{X} \leftrightarrow \underset{\text{Reversibly Adsorbed Cells}}{X_{rev}} \rightarrow \underset{\text{Irreversibly Adsorbed Cells}}{X_{irr}} \qquad (8)$$

Observation of the total amount of cells at a substratum at any one time comprises both reversibly and permanently adsorbed cells - *i.e.*, $X_t = X_{rev} + X_{irr}$.

The rigorous approach of Escher (1986) derives material balances, over the surface for each cell type, that include the rate of initial adsorption, the rate of desorption of reversibly attached cells, and the rate of transition from reversibly to permanently attached cells. For the experiments with *Pseudomonas aeruginosa* shown in Figure 5, Escher was able to estimate the individual rate dependencies for the three processes in question.

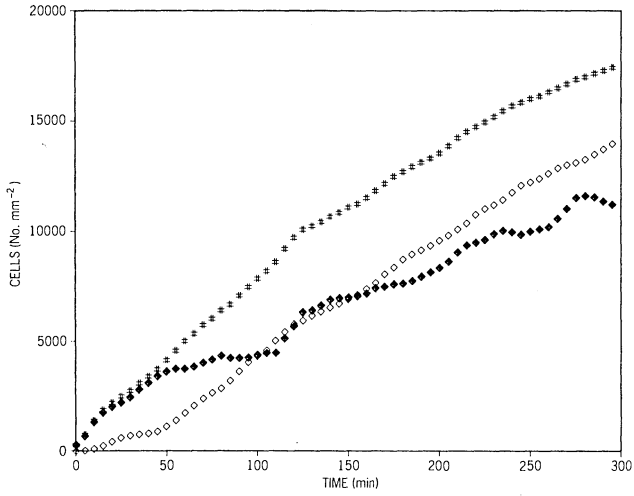


Figure 5. Progression of *Pseudomonas aeruginosa* colonization on smooth glass under laminar flow at a shear stress of 0.5 N m^{-2}. (#) sorption-related processes, desorption (◊), and (◆) net accumulation. Escher (1986).

The rate of cell adsorption (cell-$L^{-2}t^{-1}$) to the surface can be described as a first order dependency on the suspended cell concentration:

$$r_{X\text{-}ads} = k_{X\text{-}ads}X \qquad (9)$$

where $k_{X\text{-}ads}$ = cell adsorption rate constant (Lt^{-1}) and X = the suspended cell concentration (cell-L^{-3}). The adsorption rate constant, $k_{X\text{-}ads}$, is a strong function of the cell size, density difference between the cell and the bulk fluid, and the prevailing flow regime (laminar vs. turbulent). The rate of reversibly attached cell desorption, $r_{X\text{-}des}$, was found to be a discontinuous function; prior to a critical time, t_r, the rate was zero and after t_r, $r_{X\text{-}des}$, was proportional to $r_{X\text{-}ads}$. The rate of transition from reversibly to irreversibly attached cells is also a discontinuous function of time where the rate is zero prior to time t_r, and is a zero order function of the surface concentration of reversibly attached cells. From Escher's experiments, results indicate that the numbers of reversible attached cells per area is independent of time.

Another common tack is to define the actual net adsorption process, from X to X_{irr}, as a fraction of the maximum amount of cells that impacts the surface, using the concept of a *sticking efficiency*, α. The sticking efficiency is the ratio of the number of cells irreversibly attached to a substratum to the total number of cells transported to the target surface. It is often thought of as the probability that a cell, once transported to the substratum, will adsorb. Bowen *et al.* (1978) and Beal (1972) derive continuity equations describing the transport of suspended particles from a flowing fluid to the surfaces of a surrounding conduit that accounts for both convective and molecular transport mechanisms. From their work, the maximum flux of particles transported to the surface, L distance from the inlet, of a rectangular flow cell, with gap height 2h, can be written as

$$J \text{ (cells-L}^{-2}t^{-1}) = k_{tr} X \mathcal{D}_X / h \tag{10}$$

where k_{tr} = particle transport coefficient = $[(2/9)K^{0.33}/\Gamma(4/3)]$ and $K = (1/Pe)(8L/3h)$ where Pe = Peclet number = $4vh/\mathcal{D}_X$, and v = average velocity of the fluid, Γ = the gamma function ($\Gamma(4/3) = 0.89338$), $\mathcal{D}_X$ = the diffusivity of cells in the liquid. For non-motile cells, the Brownian diffusion coefficient can be estimated from the Stokes-Einstein equation,

$$\mathcal{D}_X = k_b T / 3\pi\mu d_c \tag{11}$$

where k_b = Boltzmann constant (1.38×10^{-23} J/°K), T = absolute temperature, μ = absolute viscosity, d_c = cell diameter. To compensate for cell motility, Jang and Yen (1985) propose the following equation,

$$\mathcal{D}_X = v_m d_r / 3(1 - \cos\sigma) \tag{12}$$

where v_m = velocity of motility, d_r = free length of a random run, and σ = the angle turned by the motile cell.

Rates of net adsorption are thus modeled as,

$$r_{X\text{-}dep} = \alpha J \tag{13}$$

3.1.3 *Attachment.* Attachment is the capture of particles from the fluid phase by the biofilm. This process differs from adsorption in that once cells are transported from the liquid the target surface is now a developed biofilm. The biofilm poses a different type of substratum

in that its surface morphology is generally more irregular (perhaps filamentous), very porous, more compliant, and gelatinous. Models of the rates of cell attachment simply adjust the value of the sticking efficiency α to reflect the "stickier" surface. However, recent experimental results indicate that the increased capture of cells by a biofilm-covered substratum may depend on far more subtle mechanisms than changes in mere surface morphology.

Banks (1989) and Banks & Bryers (1992) report rates of cell deposition increased when cells were exposed to biofilm surfaces versus clean glass

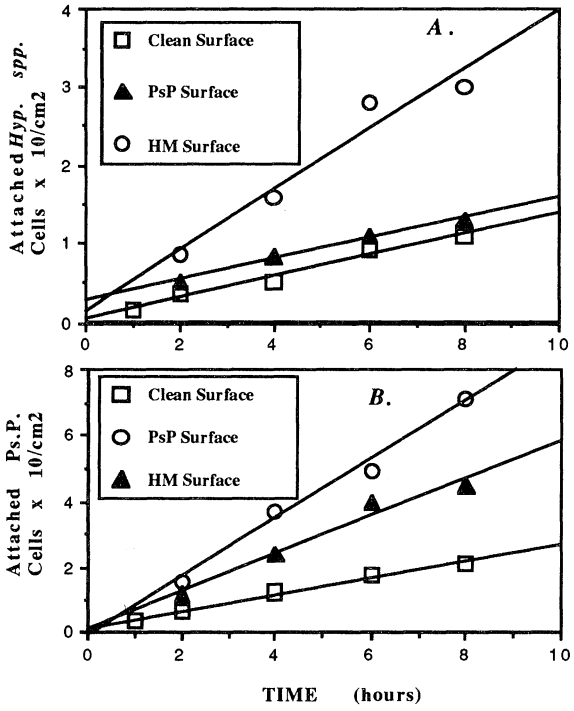


Figure 6. **(A)** Attachment of *Hyphomicrobium spp.* to various surfaces. Suspended cell concentration = 4.5E+07 cells/mL. **(B)** Attachment of *Pseudomonas putida* to various surfaces. Suspended cell concentration = 1.5E+08 cells/mL.

(Figures 6 A and B). Two species were investigated, *Pseudomonas putida* and *Hyphomicrobium ZV620* as to their deposition rates, under laminar flow conditions, onto both clean glass and biofilm-covered surfaces. *Pseudomonas putida* cells attached to a *Pseudomonas putida* biofilm at a constant rate that was zero order in attached cell concentration (0.088 cell $cm^{-2}hr^{-1}$) that was 3.6x higher than to clean glass. *Pseudomonas putida* cells attached to a *Hyphomicrobium* biofilm at a zero order in attached cell concentration (0.058 cell $cm^{-2}hr^{-1}$) that was 2.4x higher than to clean glass. *Hyphomicrobium* illustrated an attachment rate (0.041 cell $cm^{-2}hr^{-1}$) to a

Hyphomicrobium biofilm at a zero dependency on attached cells that was 3.3x higher than to clean glass. The attachment rate of *Hyphomicrobium spp.* cells to a *Pseudomonas putida* biofilm (0.016 cell $cm^{-2}hr^{-1}$) was only 1.3x that to clean glass. The attachment rate of *Hyphomicrobium cells to a Hyphomicrobium* biofilm was about half of the same rate of *Pseudomonas* cells attaching to a *Pseudomonas* biofilm, even though suspended *Pseudomonas* cell concentrations were three times higher. Results insinuate that species-dependent enhancement of cell attachment may occur by specific rather than non-specific adhesion mechanisms (Hammer & Lauffenburger, 1987).

3.2 TRANSFORMATION PROCESSES

Transformations are processes in which molecular rearrangements occur *i.e.*, reactions. Once a cell adsorbs to a surface or becomes attached to a biofilm, it will continue its metabolic processes in response to its immediate environment. Four fundamental rate processes can be identified: cellular growth and replication, product formation, endogenous decay or maintenance, and cell death and lysis. Historically, it has been easier to measure per reactor area observed transformation rates in the bulk liquid (*e.g.*, electron donor removal rate, electron acceptor uptake rate, total biofilm mass accumulation) than to measure growth, replication, and death of cells in a biofilm. This led to the development of a number of unstructured models that paid little attention to the various biofilm component parts (viable cells, total cells, extracellular polymer) or that ignore the time course of biofilm development altogether. These latter, steady-state models (Harremoës, 1971; Tanaka & Dunn, 1982) were useful at the time in estimating the flux of growth rate limiting substrate into a biofilm of fixed thickness, density, and reactivity, taking into account both internal molecular diffusion and biological reaction.

The major transformation carried out by cells in the biofilm is the metabolism of both the electron donor and acceptor to produce soluble by-products, extracellular polymers, carbon dioxide, and water. Depending on the microbial population in question and the ambient concentration of electron donor and acceptor, a biofilm can be either aerobic, anoxic (denitrifying), anaerobic (sulfate reducing bacteria, methane formers), or fermentative. In simple unstructured models, a single biological "growth rate of the biofilm", $r_B(M_X\text{-}L^{-2}t^{-1})$, relates cell growth to substrate uptake rate, r_S $(M_S\text{-}L^{-2}t^{-1})$, using a single yield coefficient, Y $(M_X\text{-}M_S^{-1})$,

$$r_S = r_B/Y \tag{14}$$

where growth rate of biomass is assumed to follow a saturation kinetic dependency on substrate concentration,

$$r_B = \mu^*SB/(K_S + S) \tag{15}$$

Said models do not take into account multiple species participating in different biological reactions, competition for the same substrate(s), or spatial gradients in substrate or cell concentration in the biofilm. For example, in the case of an anaerobic methane producing culture, one could add structure to a biofilm model by considering the various different populations involved (particulate degraders, acetic acid formers, methanogens) each with their own individual growth rate expression similar to Eqn. (15).

Analysis of biofilm bacterial metabolic rates are frequently complicated by the effects of significant mass transfer resistances in both the liquid phase and within the developing biofilm. The steady-state models (Harremoës; 1977, 1978) are useful to estimate the observed flux of growth rate limiting substrate (M_S-$L^{-2}t^{-1}$) into a biofilm of fixed thickness, density, and reactivity, taking into account both external and internal molecular diffusion coupled with a simultaneous biological reaction rate. Based on identical models for one dimensional heterogeneous catalysis, these models assume a constant biofilm concentration B (tacitly implying a spatially uniform reactivity) and diffusion path (biofilm thickness) which allows one to predict (a) the concentration profile of limiting substrate (and by stoichiometry all other nutrients) with biofilm depth and (b) the maximum substrate uptake or flux to the biofilm.

The ratio of the reaction rate observed under practical system conditions (possibly controlled by mass transfer effects) to the true instrinsic reaction rate evaluated at system conditions assuming no mass transfer effects is defined as the *effectiveness factor* for the solid (or biofilm) in question. Models by Harremoës (1977, 1978) and Vos *et al.* (1990) of immobilized cell or biofilm kinetic studies define the effectiveness factor as a function of a dimensionless group of pertinent observable system parameters. Such models define a reacting geometry (one dimensional slab, cylinder, or sphere) with uniform distribution of the biological catalyst through out the reacting volume.

For a one dimensional biofilm of thickness L and uniform areal biomass concentration B, metabolizing a growth limiting substrate S, according to a biological reaction r_g, the second order differential equation describing the concentration of S in the biofilm as a function of position is . . .

$$\mathcal{D}_e\,(d^2S(z)/dz^2) = r_g \tag{16}$$

where $\mathcal{D}_e$ = the effective diffusivity of S in the biofilm. Solution to Eqn. (16) for S as a function of the spatial coordinate z, depends upon specific system boundary conditions and the particular reaction rate dependency on local substrate concentration (*i.e.*, first or second order, or saturation kinetics). For the case of saturation kinetics, one can define the following dimensionless variables . . .

$$\kappa = S_B/K_S \quad S^* = S/S_B \quad z^* = z/L \tag{17}$$

thus rewriting Eqn. (16) as,

$$d^2S^*/d(z^*)^2 = \theta^2\, S^*/(1 + \kappa S^*) \tag{18}$$

where

$$\theta = \text{Thiele Modulus} = \left\{A_S\mu^*X_iL^2/Y_iK_S\mathcal{D}_e\right\}^{0.5} \tag{19}$$

and A_S is the surface area to volume ratio, μ^* is the maximum growth constant for the microorganism, X_i is the areal concentration of microorganisms, and K_S is the saturation constant in the growth rate expression.

Boundary conditions for the dimensionless Eqn. (18) are correspondingly,

$$S^* = 1 \quad \text{at} \quad z^* = 1$$
$$dS^*/dz^* = 0 \quad \text{at} \quad z^* = 0$$

Once the specific substrate profile is determined, the effectiveness factor can be derived according to its definition . . .

$$\eta = \frac{\text{rate observed with mass transfer}}{\text{true reaction rate without mass transfer effects}},$$

$$\eta = r_{obs}/r_{true} = \left[-\mathcal{D}_e \, dS/dz \right]_{|z=L} / \left\{-A_S \mu^* X_i L^2 S_B/(S_B + K_S)\right\} \tag{20}$$

or in dimensionless terms =

$$\eta = [(1 + \kappa)/\theta^2] \, dS^*/dz^*_{|z^*=1} \tag{21}$$

The most convenient means of depicting the influence of operating parameters on the effectiveness factor was presented by Pitcher (1975) using a modified Thiele modulus defined as,

$$\theta_P = [\theta\kappa/(1 + \kappa)] \; [2\kappa - 2ln(1 + \kappa)]^{0.5} \tag{22}$$

Classically, in heterogeneous enzyme catalysis or in steady-state biofilm models, the above derivation holds since the concentration of surface-bound catalyst (*i.e.*, enzyme or biofilm bacteria) and the diffusion path (*i.e.*, immobilized enzyme support or biofilm thickness) are assumed constant in time. Also, the reaction rate constant μ^* is assumed to pertain to one substrate conversion process.

Modifications have been made in this approach to estimate the *unsteady-state* flux of multiple growth rate limiting substrates in several biological reactions in series. Tanaka & Dunn (1982) derive four unsteady state substrate balances (ammonia, nitrite, nitrate, and oxygen) for autotrophic nitrification. This system of equations assumes a constant biofilm thickness and that the concentration of the two bacterial species involved is constant in both time and space in order to solve the set of coupled partial differential equations. A similar, steady-state model of denitrification of nitrate to nitrite then to nitrogen was presented in Boaventura & Rodrigues (1988) and Droste and Kennedy (1986). Here two separate effectiveness factors and corresponding Thiele moduli are defined for nitrate and nitrite conversion but no distinction between bacterial species is made; in essence, a uniform biofilm density is assumed constant. Unfortunately, no work has considered evaluation of an effectiveness factor for either the case of an unsteady-state biofilm thickness or time dependent concentration profiles of different bacterial species in the biofilm.

With estimates of the appropriate diffusion coefficients, a Thiele modulus and its corresponding effectiveness factor can be evaluated as biofilm thickness changes in order modify Eqn. (15) accordingly, *i.e.*,

$$r_B = \eta \; \mu^* SB/(K_S + S) \tag{23}$$

In an unstructured model, biofilm surface concentration, B, is a lumped parameter representing both biofilm bound viable cells, X_B, and extracellular polymers, EP. Extracellular polysaccharides and lipoproteins comprise the EP product pool that establishes the gelatinous matrix of the biofilm. Product formation, especially EP, is critically important in interpreting biomass yield in a biofilm system. Neglecting the amount of exogenous substrate transformed by a cell into EP will lead to an overestimation of cellular yields. Consequently, in a structured model of the biofilm formation, material balances on X_B, EP, and the limiting substrate, S would be necessary as reported by Robinson *et al.* (1984) and Turakhia and Characklis (1988). Product formation rate, r_{EP} ($M_{EP}M_X^{-1}t^{-1}$), is frequently modeled as two production terms; one growth associated production rate and one non-growth associated, traditionally term a Leudeking-Piret equation:

$$r_{EP} = k_g r_B + k_{ng} \tag{24}$$

where k_g = growth associated EP coefficient ($M_P M_X^{-1}$) and k_{ng} = non-growth associated EP coefficient ($M_{EP}M_X^{-1}t^{-1}$). Here, r_B must be modified, substituting the active biomass component X_B in place of B.

Microorganisms persist in environments that are low in essential nutrients and carbon sources. Most studies of microbial growth under oligotrophic environments observe decreasing cellular yields with decreasing growth rate. As either the limiting exogenous substrate drops below a certain value or the growth rate is low, cells turn to endogenous sources of energy - *i.e.*, maintenance. Should exogenous substrate deprevation persist for a prolonged period, the cell may cease to be metabolically active and no longer replicate; eventually the cell membrane deteriorates and lysis of the cell occurs. Collectively, these processes all detract from the net production of viable cell mass in the culture appearing as a decrease in the observed yield.

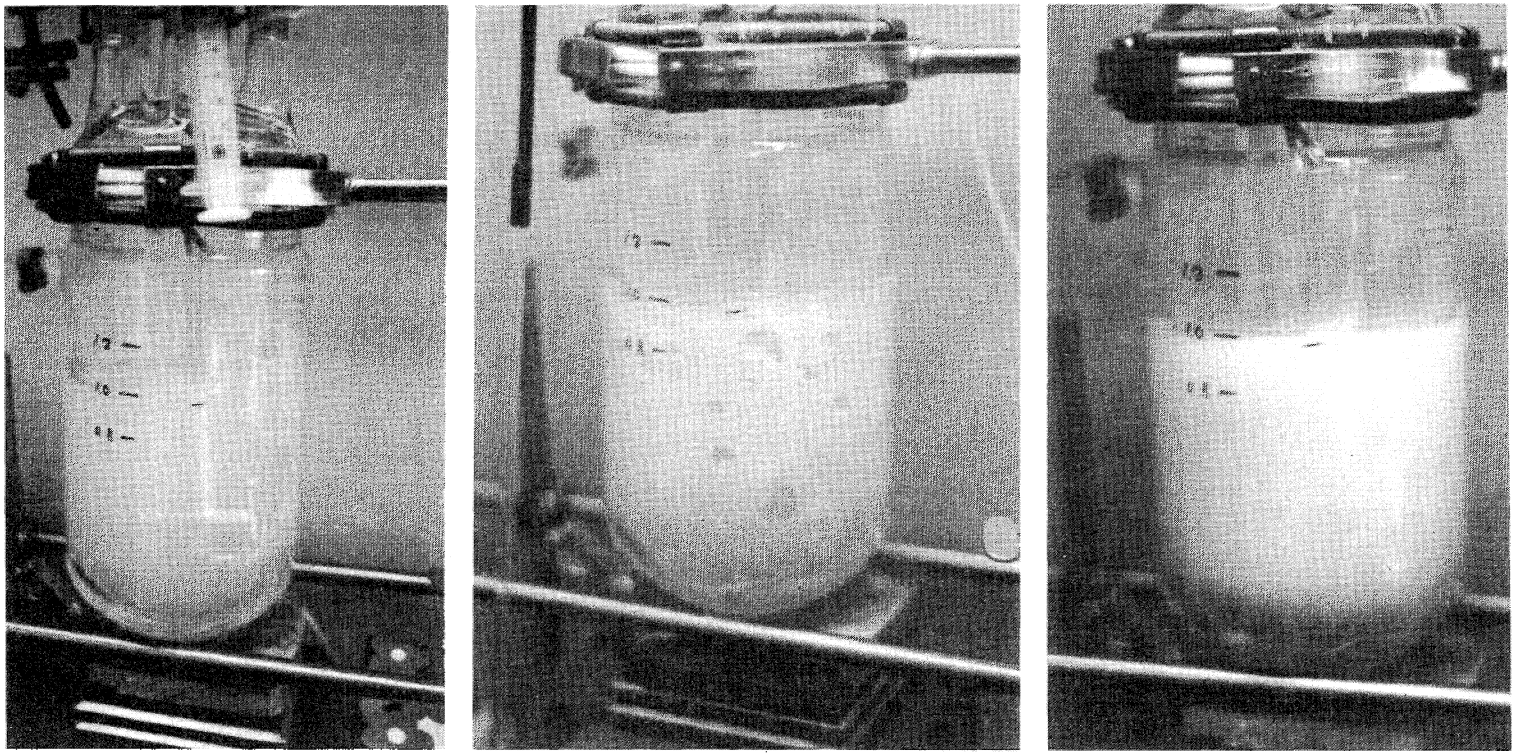
In a modeling context, the processes of maintenance, death, lysis have traditionally been lumped into a single "decay" rate that is a first order function of the amount of cell mass (either suspended or in the biofilm). Only a few researchers (Mason *et al.* 1986a,b; Banks & Bryers, 1990) have actually developed a tested structured models of microbial growth that account for the individual processes comprising decay. The most direct way to account for decay in a biofilm cell growth term is to redefine Eqn. (15) as a **net** cell growth term where,

$$r_B = [\mu^* SB/(K_S + S)] - [k_{decay}B] \tag{25}$$

where k_{decay} = endogenous decay rate constant (t^{-1}).

3.3 BIOFILM REMOVAL PROCESSES

Biofilm removal processes involve the actual loss of material from the biofilm; either cells or cell:biofilm matrix debris. Desorption (Section 3.1.2) is the release of cells from the substratum and occurs from the onset of adsorption. Biofilm detachment or removal represents loss of biomass from the biofilm and arise by way of three distinctively different mechanisms: erosion, sloughing, or abrasion.



A. Initial Biofilm Formation **B.** Isolated Sloughing of Biofilm **C.** Entire Sloughing of Biofilm

Figure 7. Sequence of biofilm formation and eventual sloughing in an anaerobic continuous bacterial culture.

3.3.1 *Abrasion.* Abrasion is the loss of biofilm due to repeat collisions between substratum particles as seen in fluidized bed reactors or in heat exchangers using cooling water of high solids content. To date, there does not exist a rate expression to describe the rate of biofilm removal by abrasion.

3.3.2. *Erosion.* Erosion is the continuous loss of biomass or cell prodigy from the upper layers of a biofilm. The rate of biofilm removal by erosion is modeled as a first order function of either biofilm amount, $r_{ero} = k_{ero}B$, where the erosion removal rate constant, k_{ero}, is a strong function of the prevailing hydraulic shear. Trulear and Characklis (1982) observed that biofilm erosion rates increased with fluid shear and biofilm amount. Applegate and Bryers (1991) reported that the growth conditions of the biofilm strongly affected biofilm removal processes. Stoichiometric limitations of carbon substrate affected biofilms that contained less EP, bound less calcium ion, and exhibited a higher erosion rate (maximum removal rate = 1.05 gm biomass carbon m^{-2} day^{-1}) than oxygen-limited biofilms that accumulated more EP, bound more calcium ions, developed rigid morphology that was very resistant to shear (maximum removal rate = 0.4 gm biomass carbon m^{-2} day^{-1}) prior to sloughing.

3.3.3. *Sloughing.* The catastrophic, apparently random, loss of large pieces or entire sections biofilm is known as sloughing. In attempting to attain a steady-state biofilm amount, the entire sloughing of a biofilm, Figure 7, can come as quite an embarrassment. All too often, random sloughing of a biofilm terminates an experiment which has had the result of yielding very little quantitative data regarding the causes of sloughing. A number of situation specific causes of sloughing have been identified: bubble formation in either anaerobic methane producing biofilm, nitrogen bubble formation in denitrifying biofilm, and artificially induced sloughing by way of calcium chelation by EGTA addition. Applegate and Bryers (1991) reported that the growth conditions of the biofilm strongly affected the biofilm removal processes of erosion and sloughing. The "fluffier" biofilm produced under carbon limited growth exhibited high erosion rates but never sloughed even when subjected to over 300 hours of nutrient starvation. Conversely, rigid biofilms cultivated under oxygen limitations showed little to no erosion but the onset of a catastrophic slough was predictable and repeatable.

4. Summary

This paper presents concepts of process analysis and mathematical modeling as applied to biofilm processes. Examples of both structured and unstructured mathematical analysis of biofilm formation and activity are given. Distinctions between homogeneous and heterogeneous reaction systems are made and errors associated with ignoring mass transfer limitations of biofilm reactions are presented. Our overall goal was to present the utility and limits of mathematical modeling of biological systems and to provide a methodology with which to systematically interpret complex biological processes such as biofilm formation.

5. References

Applegate, D. H. and Bryers, J. D. (1991) Effects of carbon and oxygen limitations and calcium concentrations on biofilm removal processes, *Biotechnol. Bioengrg.*, **37**, 17-25.

Banks, M. K. (1989) *Fate of Deposited Cells in an Aerobic Bacterial Biofilm,* Ph.D. Dissertation, Duke University, Durham, NC.

Banks, M. K. and Bryers, J. D. (1990) Cryptic growth within a binary microbial culture, *Appl. Microbiol. and Biotechnol.*, **33**, 596-601.

Banks, M. K. and Bryers, J. D. (1992) Deposition of bacterial cells onto glass and biofilm surfaces, *Biofouling*, (accepted for publication, March 1992).

Beal, S.K. (1972) Deposition of particles in turbulent flow on channel or pipe walls, *Nucl. Sci. Engrg.*, **40**, 1-11.

Boaventura, R. A. and Rodrigues, A. E. (1988) Consecutive reactions in fluidized bed biological reactors: modeling and experimental study of wastewater denitrification, *Chem. Engrg. Sci.*, **43**, 2715-2728.

Bowen, B. D., Levine, S. and Epstein, N. (1978) Fine particle deposition in laminar flow through parallel-plate and cylindrical channels, *J.Coll.Interfac.Sci.*, **54**, 375-390.

Bryers, J.D. (1980) *Dynamics of Early Biofilm Formation in a Turbulent Flow System*, Ph.D. Dissertation, Wm. M. Rice University, Houston, TX.

DePalma, V. A., Goupil, D. W. and Akers, C.K. (1979) presented at 6th Offshore Thermal Energy Conversion Conference, Washington, D.C.

Droste, R. L. and Kennedy, K. J. (1986) Sequential substrate utilization and effectiveness factors for fixed biofilms, *Biotechnol. Bioengrg.*, **28**, 1713-1720.

Escher, A. R. (1986) *Colonization of a Smooth Surface by Pseudomonas aeruginosa: Image Analysis Methods*, Ph.D. Dissertation, Montana State University, Bozeman, MT.

Hammer, D. A. and Lauffenburger, D. A. (1987) A dynamic model for receptor mediated cell adhesion to surfaces, *Biophys. J.*, **52**, 475-487.

Harremoës, P. (1977) Half-order reactions in biofilm and filter kinetics, *Vatten*, **33**, 122-143.

Harremoës, P. (1978) Biofilm kinetics, in R. Mitchell (Ed.) *Water Pollution Microbiology*, Vol. 2, J. Wiley, New York, NY, p. 82-109.

Jang, L. K. and Yen, T. F. (1985) A theoretical model of convective diffusion of motile and non-motile bacteria toward a solid surface, in *Microbes and Oil Recovery*, Vol. 1, (Zajic and Donaldson, Eds.) International Bioresources J., 226-246.

Loeb. G.I. and Neihof, R.A. (1975). Marine conditioning films, in R. E. Baier (Ed.) *Applied Chemistry at Protein Surfaces*, American Chemical Society Vol. 145, Washington, D.C. 314-335.

Mason, C. A., Bryers, J. D., and Hamer, G. (!986a) Activity, death, and lysis during microbial growth in a chemostat, *Chem. Engrg, Commun.*, **45**, 163-176.

Mason, C. A., Hamer, G., and Bryers, J. D. (1986b) The death and lysis of microorganisms in environmental processes, *FEMS Microbiol. Rev.*, **39**, 373-401.

Pitcher, W. H. (1975) Engineering of immobilized enzyme systems, *Catalyst Reviews-Science and Engineering*, **12**, 37-69.

Robinson, J.A., Trulear, M.G., and Characklis, W. G., (1984) Cellular reproduction and extracellular polymer formation by *Pseudomonas aeruginosa* in continuous culture, *Biotechnol. Bioengrg.*, **26**, 1409-1417.

Tanaka, H. and Dunn, I.J. (1982) Kinetics of biofilm nitrification, *Biotechnol. Bioengrg.*, **24**, 669-689.

Trulear, M. G. and Characklis, W. G. (1982) Dynamics of biofilm processes,*J. WPCF*, **54**, 1288.

Turakhia, M. H. and Characklis, W.C. (1988) Activity of *Pseudomonas aeruginosa* in biofilms: effects of calcium, *Biotechnol. Bioengrg.*, **33**, 406-414.

Vos, H.J., Heederik, P.J., Potters, J.J.M., and Luyben, K.C. (1990) Effectiveness factors for spherical biofilm catalysts, *Bioprocess Engineering*, **5**, 63-72.

ACKNOWLEDGEMENTS

JDB would like to thank both the National Science Foundation (BCS-9020502) and the NATO Advanced Studies Institute for their financial and intellectual support of this effort. WGC gratefully acknowledges the support of the National Science Foundation Engineering Research Centers Program and the Center's Industrial Associates.

MASS TRANSFER IN AND AROUND BIOFILMS

J. C. VAN DEN HEUVEL
University of Amsterdam
Dept. of Chemical Engineering & Biotechnological Centre
Nieuwe Achtergracht 166
1018 WV Amsterdam
The Netherlands

Introduction

Biological conversions are characterized by relatively low specific reaction rates. Many processes in which micro-organisms play a role are feasable on an industrial scale due to their potential to form aggregates spontaneously or to attach themselves as thin biofilms on a carrier material. As a consequence of these adhesive properties, it is possible to increase the volumetric conversion rate in continuously operated bioreactors by selective retention of biomass (Hamer, 1982). Furthermore, downstream processing is facilitated by the simple separation of aggregated biomass, i.e., sedimentation. These principles are succesfully applied in e.g. the activated sludge and the upflow anaerobic sludge blanket process.

Because of limitations by mass transfer from the bulk liquid to the biological particle or the simultaneous reaction and diffusion inside the particle, an optimum exists for the size of aggregates and the retention of biomass. At a given size and shape of a particle, the external mass transfer rate is determined mainly by its velocity relative to the continuous liquid phase and expressed as the Sherwood number, while the ratio between the reaction and the diffusion rate inside the particle is given by the Thiele number.

In this contribution it is aimed to substantiate the abovementioned principles governing the macro-kinetics of biofilms from a chemical engineering point of view. To this end, microbial kinetics, intraparticle mass transfer and external mass transfer will be treated to provide an operational knowledge on the scale of biofilms themselves.

Microbial kinetics

Chemical conversions are determined by three factors: thermodynamics, kinetics and transfer phenomena. Thermodynamics sets the boundary conditions for the product yield, while the reaction rate is closely related to the reaction mechanism involved. Finally, realization of a chemical conversion is obtained by tranfer of mass, heat and momentum to a reactor. The state in such a reactor is given by the balance of input, production and output, according to the various laws of conservation.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 239–250.

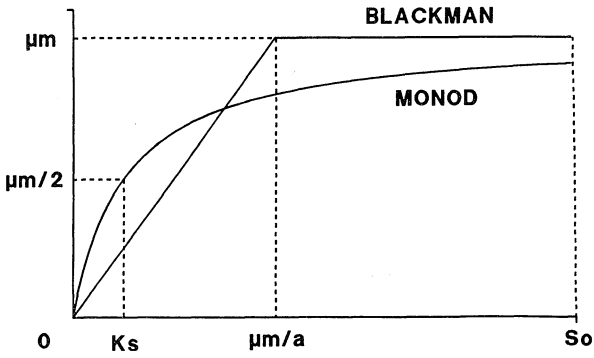


Fig. 1. *Monod and Blackman kinetics for microbial growth.*

When process conditions like temperature and pH are chosen, the thermodynamics of a microbial conversion are fixed and enter the kinetic equation as parameters like μ_m and K_s. A widely applied expression for microbial growth is given by the Monod equation:

$$\mu = \mu_m \, S/(K_s + S) \tag{1}$$

Except for extreme conditions both the biomass yield Y_x and the product yield Y_p depend linearly on the substrate consumed:

$$dX = - Y_x \, dS \qquad \text{and} \qquad dP = - Y_p \, dS \tag{2}$$

The use of Monod kinetics to form a substrate mass balance across the biofilm, however, results in a non-linear differential equation which cannot be solved analytically. Of course, numerical integration is possible, e.g. according to Runge-Kutta, but this provides less insight. Therefore, we will use Blackman kinetics which approximates the hyperbolic Monod curve by two straight lines (see Fig.1) and largely yield solvable equations.

$$\begin{aligned} \mu &= a \, S \quad \text{if} \quad S \leqslant \mu_m/a \quad \textit{first order} \\ \mu &= \mu_m \quad \text{if} \quad S \geqslant \mu_m/a \quad \textit{zero order} \end{aligned} \tag{3}$$

Using this kinetic equation enables to solve the mass balance across the biofilm, and determine the combined effect of the biological conversion and mass tranfer, i.e. the macro-kinetic behaviour.

Internal mass transfer and macro-kinetics of a flat biofilm

Diffusion is the phenomenon that the thermal motion of molecules affects a net transport which smoothes out concentration differences of each species. Diffusion can be described with Fick's law:

$$q = - D \, dS/dx \tag{4}$$

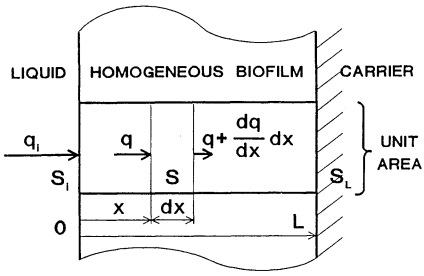


Fig. 2. *Flux in a flat biofilm; nomenclature.*

In every point in the biofilm substrate is removed at a rate r, and in the stationairy state this will equal the decrease of the flux with depth (see Fig.2):

$$r = \mu\ X/Y_x = -\ dq/dx \tag{5}$$

Combination of equations (4) and (5) gives the mass balance over a thin layer dx in the biofilm:

$$D_e\ d^2S/dx^2 - r = 0 \tag{6}$$

D_e indicates the effective diffusion coefficient of the substrate in the biofilm. It depends on the density and structure of the biofilm (e.g. porosity and tortuosity), and its value typically amounts to 70% of the value in the liquid phase. To increase the usefulness of equation (6) and its solutions, dimensionless equations are often used in chemical engineering by normalizing with respect to characteristic system parameters:

$$s = S/S_i \qquad \text{and} \qquad \xi = x/L \tag{7}$$

By substituting (7) into (6) we obtain a dimensionless mass balance:

$$\frac{d^2s}{d\xi^2} = \frac{rL^2}{D_e S_i} \qquad \text{with boundary conditions:} \quad \begin{array}{l} \xi = 0 \rightarrow s = 1 \\ \xi = 1 \rightarrow ds/d\xi = 0 \end{array} \tag{8}$$

The requirement that $ds/d\xi = 0$ at the carrier interface, follows from the fact that no transport occurs to the surface itself.

ZERO ORDER KINETICS

To solve (8) we will consider first the simple case of zero order kinetics when $r \equiv r_m = \mu_m X/Y_x$, and we obtain:

$$\frac{d^2s}{d\xi^2} = \frac{r_m L^2}{D_e S_i} = \varphi^2 \qquad \text{with} \qquad \varphi = L\left[\frac{r_m}{D_e S_i}\right]^{\frac{1}{2}} = L\left[\frac{\mu_m X}{Y_x D_e S_i}\right]^{\frac{1}{2}} \tag{9}$$

The dimensionless constant φ is the Thiele number (or modulus), and represents the ratio of the reaction rate and the diffusion rate in the biofilm. If $\varphi > 1$ the system is diffusion limited, conversely, when $\varphi < 1$ it is reaction limited. The solution of (9) is obtained by integration between the boundary conditions (complete derivation only as an example):

$$\frac{d^2s}{d\xi^2} = \frac{d}{d\xi}\left(\frac{ds}{d\xi}\right) = \varphi^2 \quad \rightarrow \quad \int_{ds/d\xi}^{0} d\left(\frac{ds}{d\xi}\right) = \int_{\xi}^{1} \varphi^2 d\xi \quad \rightarrow$$

$$\left[\frac{ds}{d\xi}\right]_{ds/d\xi}^{0} = \varphi^2 \left[\xi\right]_{\xi}^{1} \quad \rightarrow \quad 0 - \frac{ds}{d\xi} = \varphi^2(1 - \xi) \quad \rightarrow \qquad (10)$$

$$\int_{1}^{s} ds = \varphi^2 \int_{0}^{\xi} (\xi - 1)\, d\xi \quad \rightarrow \quad \left[s\right]_{1}^{s} = \varphi^2 \left[\tfrac{1}{2}\xi^2 - \xi\right]_{0}^{\xi}$$

$$\rightarrow \quad s = \varphi^2(\tfrac{1}{2}\xi^2 - \xi) + 1 \quad \rightarrow \quad s = \frac{\varphi^2}{2}\xi^2 - \varphi^2\xi + 1 \qquad (11)$$

This solution is presented graphically for various values of φ in Fig. 3, and has only meaning for $s > 0$, i.e. $\varphi < \sqrt{2}$ when the biofilm is comletely penetrated. With Blackman kinetics this pure zero order case is applicable when S_i is so large and L so small that the substrate concentration at the carrier interface satisfies $S_L > \mu_m/a$.

For a partially penetrated biofilm and $\varphi > \sqrt{2}$ we have to change the second boundary condition into:

$$\xi = \xi' < 1 \quad \rightarrow \quad s = 0 \; ; \; ds/d\xi = 0 \qquad (12)$$

Note that with pure zero order kinetics the conversion always proceeds at maximal speed, as a consequence the substrate concentration may indeed attain a value of zero. This situation will not occur with Blackman kinetics, since the 'tailing' first order kinetics for $S < \mu_m/a$ only *approaches* zero (see further). Using the new boundary conditions (12) we obtain a slightly different solution for (8) (see Fig. 3):

$$\xi' = \frac{\sqrt{2}}{\varphi} \qquad s = \frac{\varphi^2}{2}\xi^2 - \varphi\sqrt{2}\,\xi + 1 \qquad (13)$$

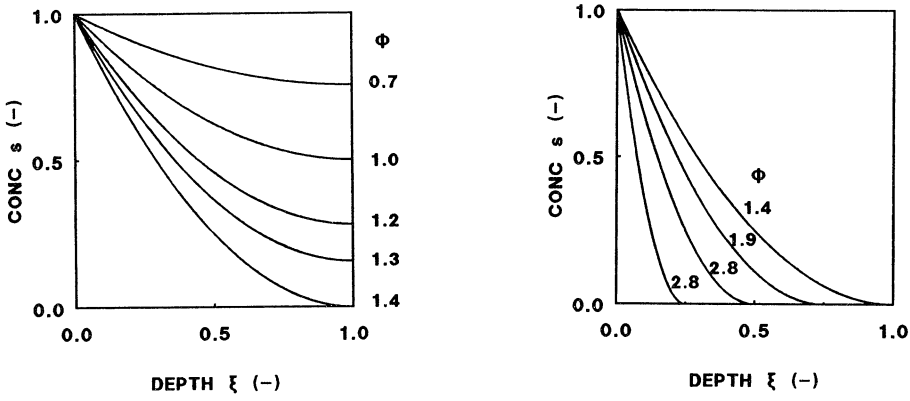


Fig. 3. *Dimensionless concentration profiles for a completely penetrated flat biofilm with zero order kinetics at several values of the Thiele modulus φ (left); idem, partially penetrated film (right).*

It should be realised that part of the biofilm lying deeper than the (relative) penetration ξ' is completely inactive. *In situ* measurements with micro-electrodes indicate that inactive zones are the rule, rather than the exception (De Beer et al., 1990; Sweerts et al., 1990; De Beer et al., 1991).

The total conversion by the biofilm (per unit area!) q_i is of course given by:

$$q_i = - D_e \frac{dS}{dx}\Big|_{x \downarrow 0} = - D_e \frac{S_i}{L} \frac{ds}{d\xi}\Big|_{\xi \downarrow 0} \qquad (14)$$

In the case of a completely pentrated biofilm we obtain from (10):

$$q_i = - D_e \frac{S_i}{L} \varphi^2 \left[1 - \xi\right]_{\xi \downarrow 0} = r_m L \qquad (15)$$

while in case of a partially penetrated biofilm:

$$q_i = r_m L \xi' = \left[2 r_m D_e S_i\right]^{\frac{1}{2}} \qquad (16)$$

This total conversion rate q_i is important for the macro-kinetic behaviour of the biofilm. In this context the efficiency η of the biofilm is defined as the ratio between the actual conversion rate and the theoretical conversion rate if no limitation by diffusion would occur at all. For a flat biofilm and pure zero order kinetics it is easily obtained that:

complete penetration	$\varphi < \surd 2$	and	$\eta = 1$
partial penetration	$\varphi > \surd 2$	and	$\eta = \xi' = \surd 2/\varphi$

This means that in this specific case there is a very simple relation between the efficiency η and the Thiele modulus φ.

FIRST ORDER KINETICS

After this somewhat extensive treatment of zero order kinetics we will examine another simple case when the substrate concentration at the liquid/biofilm interface $S_i < \mu_m/a$, as a consequence we are dealing with pure first order kinetics: $r = kS = [aX/Y_x]S$. The mass balance (8) over the biofilm is then given by:

$$\frac{d^2s}{d\xi^2} = \varphi_1^{\,2}\, s \qquad \text{with:} \qquad \varphi_1 = L\left[\frac{k}{D_e}\right]^{\frac{1}{2}} = L\left[\frac{aX}{Y_x D_e}\right]^{\frac{1}{2}} \qquad (17)$$

and boundary conditions: $\xi = 0 \rightarrow s = 1$

$\xi = 1 \rightarrow ds/d\xi = 0$

Note that the first order Thiele modulus φ_1 is defined differently to retain the same physical meaning. The solution of this equation is less simple and given by:

$$s = \frac{\cosh(1 - \xi)\varphi_1}{\cosh \varphi_1} \qquad (18)$$

A number of these concentration profiles is given in Fig. 4. From equation (14) and differentiation of (18), one obtains the total conversion of the biofilm:

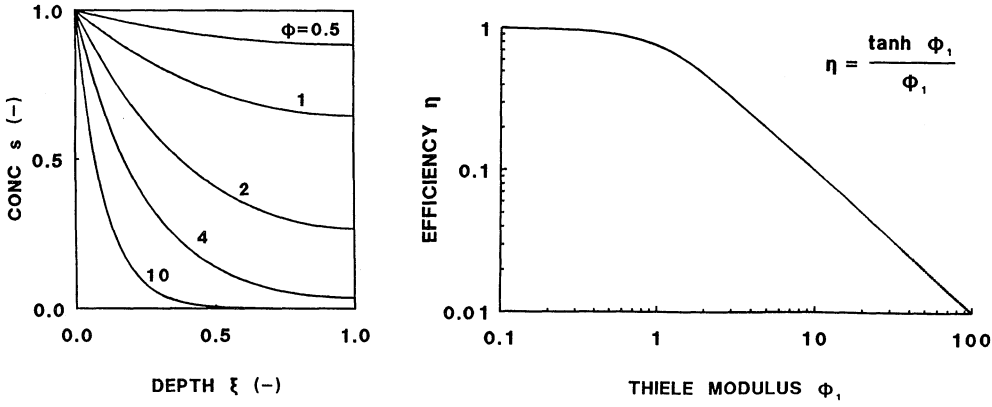


Fig. 4. *Dimensionless concentration profiles in a flat biofilm with first order kinetics at several values of the Thiele modulus φ (left); concomitant efficiency as a function of the Thiele modulus, linear relation if φ > 2 (right).*

$$q_i = r\, S_i\, L\, \frac{\tanh \varphi_1}{\varphi_1} = r\, S_i\, L\, \eta \qquad (19)$$

Since the maximal conversion rate at infinitely fast diffusion in this case equals rS_iL, equation (19) also presents the expression for the efficiency η (see Fig. 4).

BLACKMAN KINETICS

Finally, we have to combine both types of kinetics for the partially penetrated biofilm. At a certain distance ξ_B in the biofilm the substrate concentration reaches the value $S = \mu_m/a$ where, according to the Blackman approximation, zere order changes into first order kinetics (see Fig. 5). Until this point (8) holds, while afterwards (17) is in force, however, both with other boundary conditions:

$S > \mu_m/a$: $\xi = 0 \rightarrow s = 1$; $\xi = \xi_B \rightarrow s = s_B = \mu_m/a.S_i$ — *zero order*

$S < \mu_m/a$: $\xi = \xi_B \rightarrow s = s_B$; $\xi = 1 \rightarrow ds/d\xi = 0$ — *first order*

Using these we get somewhat different solutions:

$$s = \tfrac{1}{2}\varphi^2\, \xi^2 + \alpha\, \xi - [\, \tfrac{1}{2}\varphi^2\, \xi_B{}^2 + \alpha\, \xi_B\,] + s_B$$

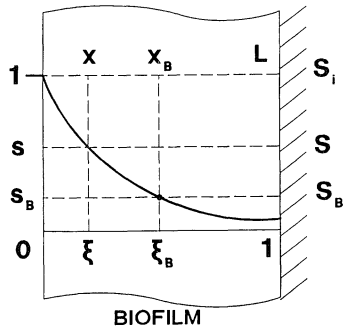
with: $$\xi_B = \frac{[\, \alpha^2 - 2\varphi^2(1 - s_B)\,]^{\frac{1}{2}} - \alpha}{\varphi^2}$$

zero order (20)

$$s = s_B\, \frac{\cosh(1 - \xi)\varphi_1}{\cosh \varphi_1}$$

first order (21)

The integration constant α from (20) can be solved in principle, since there is an additional requirement that the slopes of (20) and (21) in point ξ_B are identical.



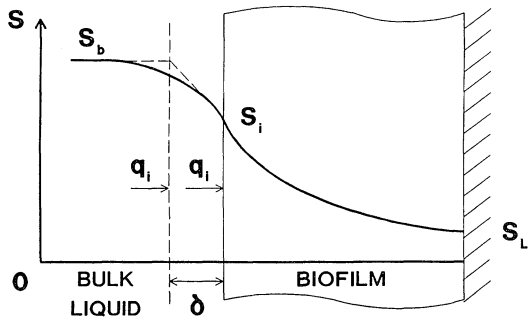


Fig. 5. *Transition from zero to first order kinetics; nomenclature.*
Fig. 6. *Concentration profile and flux in the liquid boundary layer.*

Physically this means that mass transfer should be continuous in this point, see (4):

$$\frac{ds}{d\xi}\Big|_{\xi=\xi_B} = \varphi^2\xi_B + \alpha = \frac{\varphi_1 s_B}{\cosh\varphi_1}\sinh(1-\xi_B)\varphi_1 \qquad (22)$$

Unfortunately, α can not be written explicitely and (22) has to be solved by numerical itteration, e.g. according to Newton-Raphson.

Summarizing conversion in biofilms according to Blackman kinetics, and depending on the substrate concentration at the liquid and/or carrier interface, we may distinguish:

$S_i >>$	and	$S_L > \mu_m/a$	→	pure zero order
$S_i > \mu_m/a$	but	$S_L < \mu_m/a$	→	no analytical solution
$S_i < \mu_m/a$			→	pure first order

As a consequence, the advantage of simple Blackman kinetics cancels in the case of intermediate concentrations. Therefore, in those cases we can use Monod kinetics (or more complicated kinetics including inhibition) as well, and solve the mass balance by numerical integration.

External mass transfer

So far the substrate concentration at the biofilm-liquid interface S_i has been used deliberately, and not the concentration in the bulk liquid S_b as often encountered in the literature. The adjacent liquid, however, can not move freely with respect to the interface. Therefore, the (local) bulk concentration is found only at some distance from the biofilm, depending on the hydrodynamic regimen and the biofilm geometry (see Fig. 6). It should be noted that, although bulk concentrations can be defined on the scale of the biofilm thickness, substrate gradients usually will be found in axial and / or radial direction on the scale of the bioreactor.

External mass transfer is formulated as the product of a conductivity parameter and the driving force, and should equal the flux q_i at the biofilm interface:

$$q_i = - D_e \frac{dS}{dx}\Big|_{x \downarrow 0} = k_l (S_b - S_i) \qquad (23)$$

The mass transfer coefficient k_l is an overall conductivity parameter comprising the contributions of diffusion, natural and forced convection. In a further simplification, the film model assumes an ideally mixed bulk phase and a completely stagnant boundary layer at the biofilm interface. All mass transfer resistances are thought to be located in this diffusive boundary layer of uniform (hypothetical) thickness δ (see Fig. 6), and therefore:

$$k_l = D/\delta \qquad (24)$$

In a number of specific cases exact solutions for k_l are at hand, otherwise empirical correlations have been developed for the different flow conditions and catalyst / reactor geometries. More detailed information can be found in text books on physical transport phenomena (e.g. Bird et al., 1960). In this contribution we will consider a spherical biofilm suspended in the liquid phase, as encountered in e.g. fluidized bed and gas-lift reactors. An appropriate (dimensionless) correlation to be used is the widely applied equation of Ranz and Marshall (1952):

$$\frac{k_l d}{D} = 2 + 0.6 \left[\frac{v_s \rho_l d}{\eta_l}\right]^{\frac{1}{2}} \cdot \left[\frac{\eta_l}{\rho_l D}\right]^{\frac{1}{3}} \quad \text{or} \quad Sh = 2 + 0.6\, Re^{\frac{1}{2}} \cdot Sc^{\frac{1}{3}} \qquad (25)$$

The Sherwood number relates total mass transfer and diffusive mass transfer. This equation predicts Sh = 2 for a motionless fluid; this asymptote can be derived theoretically and is confirmed by experiments. Sh $\rightarrow \infty$ would be required for $S_i \rightarrow S_b$. The Reynolds number for the biological particle characterises the surrounding liquid flow, and contains the slip velocity v_s and the diameter d of the particle. The Schmit number relates the diffusion of momentum and mass, and contains physical constants of the liquid phase.

The slip velocity may be approximated by the settling velocity; for a spherical particle and Re $< 10^3$:

$$v_s = \frac{g}{18} \frac{X - \rho}{\eta_l} d^2 \qquad (26)$$

Defining a length co-ordinate *from* the centre of the sphere *to* the interface $\sigma = 2x/d$, and a concentration normalised on the *bulk* concentration $c = S/S_b$, the microscopic mass balance of a spherical particle with Monod kinetics becomes:

$$\frac{d^2c}{d\sigma^2} + 2 \frac{dc}{d\sigma} - \frac{Kc}{1 + Mc} = 0 \qquad \text{with:} \qquad K = \frac{\mu_m X d^2}{4 Y_x D_e K_s} \qquad M = \frac{S_b}{K_m} \qquad (27)$$

and boundary conditions: $\sigma = 0 \rightarrow dc/d\sigma = 0$

$\sigma = 1 \rightarrow c = c_i$

Compared to the flat biofilm, the second diffusion term in the mass balance represents the additional 'focussing' effect due to the spherical geometry. Rewriting the continuity requirement from (23) in the new dimensionless variables, and using the definition of Sh we obtain:

$$c_i = 1 - \frac{D_e}{D}\frac{2}{Sh}\frac{dc}{d\sigma}\Big|_{\sigma=1} = 1 - \frac{1}{Bi}\frac{dc}{d\sigma}\Big|_{\sigma=1} \qquad (28)$$

The procedure to solve (27) is as follows: establish K, M and Sh from physical constants, kinetic parameters, the particle density and diameter, and the bulk substrate concentration. Guess the concentration in the centre and calculate the concentration gradient by numerical integration of (27), then check whether c_i thus found satisfies (28). Adjust the initial guess untill the solution is obtained, i.e. the concentration difference over the boundary layer ($1 - c_i$) matches the flux ($dc_i/d\sigma$).

These calculations were done at different Sh numbers, and taking equal diffusion coefficients in the particle and the liquid phase (Huisman et al., 1990). The results are presented in Fig. 7, in which the effectiveness factor η is plotted against a generalized Thiele number for Monod kinetics according to Moo-Young & Kobayashi (1972):

$$\varphi_M = \frac{M}{3(M+1)} \cdot \left[\frac{K}{2\{M - ln(M+1)\}}\right]^{\frac{1}{2}} \qquad (29)$$

As mentioned before, Thiele numbers for biocatalyst particles are often presented assuming $C_i = C_b$, i.e. in the case of Sh = ∞. The calculated efficiency in Fig. 7, however, shows that this procedure is highly uncertain as the external mass transfer rate considerably influences the effectiveness factor for Thiele numbers over unity. The generalized Thiele number for different biological sytems were estimated from literature data, and inserted in Fig. 7 (Huisman et al., 1990). The maximal effect of external mass transfer, amounting to a factor of 5, is to be expected in the case of highly reactive sytems like denitrifying and acidogenic aggregates. About 25–50% of this effect can be realized by suspending these biomass particles in, for example, fluidized bed reactors, since a Sh number of 10–25 can be estimated for such systems.

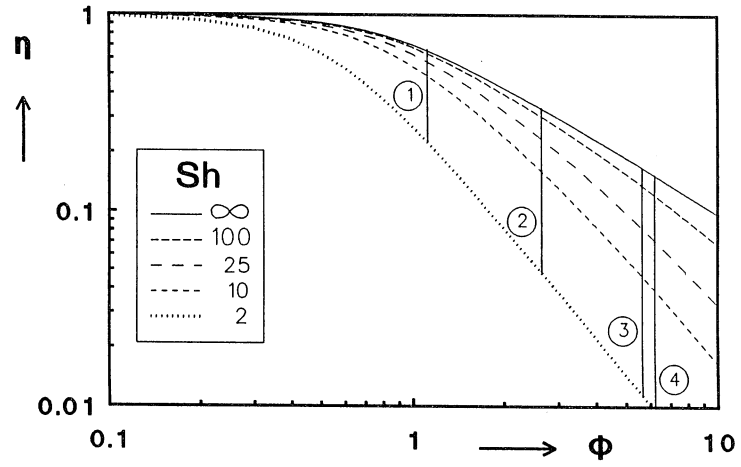


Fig. 7. *Efficiency versus Thiele modulus φ_M for various values of Sh. Indicated are estimated values of φ_M for 3 mm biocatalyst particles of 1) methanogenic bacteria, 2) immobilized yeast cells, 3) denitrifying bacteria, and 4) acidifying bacteria.*

Discussion

All kinetic equations for microbial growth are in fact of a phenomenological nature. Their value is determined by the accuracy and ease to describe experimental observations. The choise of the slope (a) in Blackman kinetics can be adapted to the circumstances (see e.g. Harremoës, 1976), and in most cases the difference with Monod kinetics is negligible. In sysems with selective retention of biomass both equations are even inappropriate, since they do not account for death or lysis of organisms. Especially in thicker biofilms, lysis plays an important role and causes disruption of aggregates and sloughing of complete layers.

Another important point not considered yet is growth. By using only substrate balances, the concomitant biomass increase has not been accounted for. Contrarily, homogeneous biofilms with a constant thickness were assumed. In reality, a constant amount of biomass in a reactor is a *dynamic* system in which (i) newly formed biomass is removed by attrition from the biofilm surface and enters the effluent stream, or (ii) growth is just balanced by lysis and the net growth is equal to zero (Beeftink and Van den Heuvel, 1990). If net biomass growth is undesirable, the latter argument is sometimes used to justify simplifying assumptions. However, this kind of reasoning in not correct, since growth and lysis occur at different places in the biofilom. As a result, the biofilm density will decrease with depth (Beeftink and Van den Heuvel, 1988; Pereboom et al., 1988).

Inhomogeneous distributions may also occur with respect to kinetics. Depending on the local substrate concentration, micro-organisms may change their metabolic routes and the associated ATP yield, as in the well known Crabtree effect. Furthermore, the higher product concentrations in the biofilm (profiles complementary to the substrate profile) may influence μ_m in (i) a general way through the concomitant change in local pH (Zoetèmeyer et al., 1982; De Beer and Van den Heuvel, 1988; De Beer et al., 1992), or (ii) more specifically by some inhibitory mechanism (Van den Heuvel and Beeftink, 1988; Van den Heuvel et al., 1988). As a rule, all these complications result in a lower efficiency as compared to the homogeneous and static biofilm. Only in the case of substrate inhibition, e.g. phenol oxidation, lower substrate concentrations due to transport limitations may lead to higher conversion rates, and conceivably η may be larger than 1.

In this contribution we have limited ourselves to diffusive mass transfer in the biofilm and the film theory. Potentially, convective mass transfer is able of increasing transport rates some 3-4 orders of magnitude, and offers challanging ways of increasing biomass efficiency. Convective flows through a macroporous biofilm have been reported in a number of cases, and may be accomplished by pressure gradients (Wittler et al., 1986), pressure fluctuations (Van den Heuvel et al., 1992), and inertial forces (Bringi and Dale, 1990). Cultivation techniques to obtain the required macroporous structure as well as *in vivo* characterization methods (e.g. NMR analysis, Cotts, 1991; Lewandowski, 1991) are new and intrigueing research areas.

Reduction of the external mass transfer resistance by intensive bulk liquid mixing will be limited by disruption of the particles and severe reduction of the biomass retention. However, *local* mixing of the boundary layer by natural convection, as occuring with gaseous end products, could contribute significantly (Huisman et al., 1990). Convective flows induced by (i) gas bubbles rising from the biofilm surface, as well as (ii) the smaller density of the substrate depleted liquid, will enhance mass transfer. Especially in fixed film reactors, accelleration of external mass transfer could contribute

significantly, as gas bubbles are able to travel in the diffusive boundary layer for a considerable distance, viz. along the surface of vertically oriented biofilms.

It might be speculated that convective contributions to mass transfer have so far gone unnoticed (Logan and Hunt, 1988), and were lumped into apparent microkinetic parameters of the biofilm or active biomass density.

From these arguments it may be appreciated that a conceptual understanding of all phenomena occuring in biofilms, is extremely important. Secondly, these phenomena and their interactions are so complicated that modelling only is of limited value. Therefore, I would like to state as a final conclusion, that actual measurement of substrate and product concentration profiles inside biofilms with appropriate sensors is required for a proper design, scaling-up and operation of biofilm reactors.

Nomenclature

a	slope Blackman kinetics	$m^3/kg.s$
c	concentration S/S_b	–
d	particle diameter	m
D	diffusion coefficient	m^2/s
g	gravity accelleration	m^2/s
k	reaction rate constant	s^{-1}
k_l	mass transfer coefficient	m/s
K	constant, see text	–
K_s	saturation constant	kg/m^3
L	thickness biofilm	m
M	constant, see text	–
q	mass flux	$kg/m^2.s$
r	reaction rate per volume	$kg/m^3.s$
Re	Reynolds number	–
s	concentration S/S_i	–
S	substrate concentration	kg/m^3
Sc	Schmitt number	–
Sh	Sherwood number	–
v_s	slip velocity	m/s
x	length co-ordinate	m
X	biomass density	kg/m^3
Y_p	product yield	kg/kg
Y_x	biomass yield	kg/kg

Greek

η	dynamic viscosity	Pa.s
ξ	length from interface	–
ρ	density	kg/m^3
φ	(0 order) Thiele number	–
σ	length from centre	–
δ	thickness boudary layer	m
μ	specific growth rate	s^{-1}

Subscripts

b	bulk
B	Blackman order change
e	effective
i	interface
l	liquid phase
m	maximal
M	Monod kinetics
1	first order kinetics

References

Beeftink H.H, Van den Heuvel J.C. (1988) *Physical properties of bacterial aggregates in a continuous-flow reactor with biomass retention.* In: Granular anaerobic sludge; microbiology and technology, Lettinga G, Zehnder A.J.B, Grotenhuis J.T.C, Hulshoff Pol L.W. (eds.) Pudoc, Wageningen, 162-169

Beeftink H.H, Van den Heuvel J.C (1990) *Aggregates of various and varying size and density: a model for a continuous flow reactor,* Chem. Eng. J, **44**, B1-B13

Bird R.B, Stewart W.E, Lightfoot E.N. (1960) *Transport phenomena*, Wiley, New York.

Bringi V, Dale B.E. (1990) *Experimental and theoretical evidence for convective nutrient transport in an immobolized cell support*. Biotechnol. Progr, **6**, 205–209

Cotts, R.M. (1991) *Diffusion and diffraction*, Nature, **351**, 443–444

De Beer D, Van den Heuvel J.C. (1988) *Gradients in immobilized biological systems*. Anal. Chim. Acta, **213**, 259–265

De Beer D, Van den Heuvel J.C, Sweerts J.P.R.A. (1990) *Microelectrode studies in immobilized biological systems*. in "Physiology of Immobilized Cells", De Bont J.A.M, Visser J, Mattiasson B, Tramper J. (eds), Elsevier Science Publishers, Amsterdam, 613–624

De Beer D, Sweerts J-P.R.A, Van den Heuvel J.C. (1991) *Microelectrode measurement of ammonium profiles in freshwater sediments*, FEMS Microbiol. Ecol, **86**, 1–6

De Beer D, Huisman J.W, Van den Heuvel J.C, Ottengraf S.P.P. (1992) *The effect of pH profiles in methanogenic aggregates on the kinetics of acetate conversion,* Water Res, *in press*

Hamer, G. (1982). *Recycle in fermentation processes*. Biotechnol. Bioeng. **24**, 511–531.

Harremoës, P. (1976). *The significance of pore diffusion to filter denitrification*. J. Water Pollut. Control Fed. **48**, 377–388.

Huisman J.W, Van den Heuvel J.C, Ottengraf S.P.P. (1990) *Enhancement of external mass transfer by gaseous end products*, Biotechnol. Progr, **6**, 425–429

Lewandowski Z. (1991) Montana State University, Bozeman, USA, *pers. comm.*

Logan B.E, Hunt J.R. (1988) *Bioflocculation as a microbial response to substrate limitations*, Biotechnol. Bioeng, **31**. 91–101

Moo-Young M, Kobayashi T. (1972) *Effectiveness factors for immobilized-enzyme reactions*. Can. J. Chem. Eng, **50**, 162–167

Pereboom J.H.F, Van den Heuvel J.C, Ottengraf S.P.P. (1988) *Physical characterization of biocatalyst particles*. Proc. 2nd Neth. Biotechnol. Congress, Breteler H, Van Lelyveld P.H, Luyben K.Ch.A.M. (eds), Neth. Biotechnol. Soc. / TNO, Zeist, 308–313

Ranz W.E, Marshall W.R. (1952) *Evaporation from drops I*, Chem. Eng. Progr, **48**, 141–146

Sweerts J-P.R.A, De Beer D, Nielsen L.P, Verdouw H, Van den Heuvel J.C, Cohen Y, Cappenberg T.E. (1990) *Denitrification by sulfur oxidizing* Beggiatoa *ssp. mats on freshwater sediments,* Nature, **344**, 762–763

Van den Heuvel J.C, Beeftink H.H. (1988) *Kinetic effects of the simultaneous inhibition by substrate and product*. Biotechnol. Bioeng, **31**, 718–724

Van den Heuvel J.C, Beeftink H.H, Verschuren P.G. (1988) *Inhibition of the acidogenic dissimilation of glucose in anaerobic continuous cultures by free butyric acid*. Appl. Microbiol. Biotechnol, **29**, 89–94

Van den Heuvel J.C, Vredenbrecht L.H.J, Ottengraf S.P.P. (1992) *Acceleration of mass transfer by pressure oscillations*, Proc. 4th Neth. Biotechnol. Congress, Balkema Publishers, Rotterdam, The Netherlands, F25

Wittler R, Baumgartl H, Lübbers W, Schügerl K. (1986) *Investigations of oxygen transfer into* Penicillium chrysogenium *pellets by microprobe measurements*. Biotechnol. Bioeng, **28**, 1024–1036

Zoetemeyer R.J, Van den Heuvel J.C, Cohen A. (1982) *pH-Influence on acidogenic dissimilation of glucose in an anaerobic digestor*. Wat. Res, **16**, 303–311

BIOFILM MODELLING : STRUCTURAL, REACTIONAL AND DIFFUSIONAL ASPECTS

B. CAPDEVILLE
K.M. NGUYEN
J.L. ROLS
National Institute of Applied Sciences
Department of Industrial Processes Engineering
Unité de Recherche Traitement Biologique
Complexe Scientifique de Rangueil
31077 Toulouse Cédex
France

1. Introduction

If we have to optimize biological wastewater treatment processes using fixed mixed cultures and determine their size and capacity, it is necessary to know the kinetics of substrate removal and biofilm growth as these are the tools for establishing matter balances. For several years, the literature has shown that biofilms can be characterized by an active thickness beyond which the growth substrate becomes limiting. This thickness is generally estimated from mathematical models combining transport by diffusion and more or less complex reaction kinetics (Atkinson, 1974 ; La Motta, 1976 ; Williamson et al., 1976 ; Harremoes, 1977 ; Grady et al., 1980 ; Rittman, 1982 ; Characklis et al., 1990 ; etc.). Moreover, Sanders' (1966) preliminary work and much more recent experimental studies using micro-electrodes (Revsbech, 1989) have shown the presence of dissolved oxygen concentration gradients within biofilms.

However, a number of experimental results obtained with thin biofilms (thickness less than 40 μm) in perfectly controlled reactors (Nouvion, 1985 ; Lertpocasombut, 1991) have shown that the biological activity of these systems is not maximal and the micro-organisms composing the biofilm are not all fully active. This leads us to presume that physical (shear stress, etc.) and biological (metabolite inhibition, etc.) phenomena may also occur while the biofilm is maturing. But these phenomena are not taken into account at present in the conventional concept, in which only resistance to transfer by diffusion is used. And yet these phenomena have been shown to exist in thin, perfectly homogeneous biofilms used in a three-phase fluidized bed reactor with suitable supports (controlled hydrodynamics). As an example, the first part of figure 1 (airflow 5.7 m/h) shows that the accumulation of biofilm observed around the support is accompanied by a decrease in the active fraction of the fixed bacteria from 85 to 50%. Increasing the airflow to 10.2 m/h causes part of the biofilm to become detached and the active fraction of the bacteria to increase from 50 to 80%. It should be noted that, for these two operating conditions, the quantity of substrate eliminated is the same, which indicates that, although the thicknesses are different, both the types of biofilm developed have the same reactivity with respect to the liquid phase. We shall come back to these results later in the article.

In order to gain a better understanding of the phenomena likely to influence the reactivity of a biofilm (made up of a mixed population of bacteria breaking down complex substrates such as can be found in urban wastewater), we performed two studies, on aerobic and anaerobic films. The initial intention of this research was to verify and validate the hypotheses of a conventional

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 251–276.

modelling approach using the diffusion concept. The main hypotheses are the following :

. the biofilm has the form of a plane, homogeneous surface ;

. a permanent regime has been established with respect to the thickness of the film ;

. transport by diffusion in the film is in one direction ;

. the growth kinetics of the fixed micro-organisms are identical to those characterizing free mixed cultures (0, 1st, etc. order, Monod, Contois ...).

A reactor was designed specially for the study and the biofilm observation and measurement methods were adapted to the analysis and control of the kinetic laws (Belkhadir, 1986 ; Nguyen, 1989).

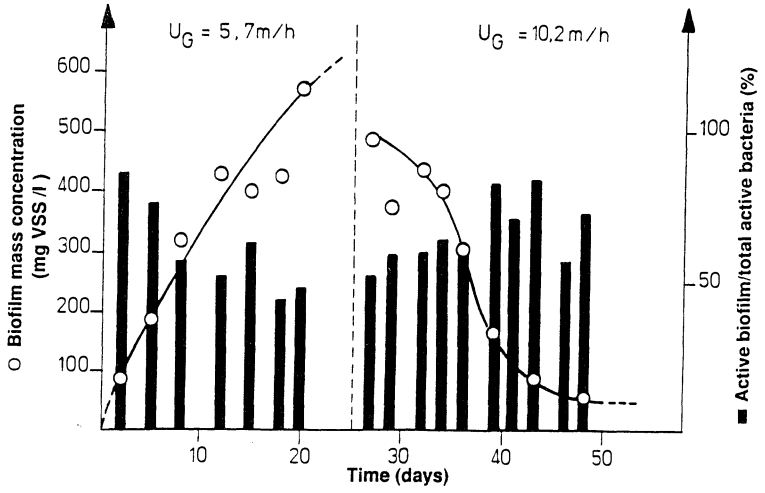


Figure 1. Behaviour of the mass of thin biofilm and micro-organism activity for two different sets of operating conditions in a three-phase fluidized bed reactor (Lertpocasombut, 1991). The activity percentage is expressed by the ratio between the activity of biofilm and the activity of total bacteria in the reactor determined by INT method (Nguyen, 1985).

2. Materials and methods

2.1. CHOICE OF REACTOR

Our criteria for reactor design were the following : control of the hydraulic stresses, which must act in a uniform and constant manner on the biofilm ; monitoring the stirring of the medium so as to have a perfectly mixed flow ; ease of sampling and of measurement of the values characteristic of the biofilm, particularly its mass. The first two points are solved in a ring reactor composed of two coaxial cylinders, one of which is mobile. Such reactors have been used by Kornegay et al. (1968), La Motta (1976), Williamson et al. (1976), and Trulear et al. (1982). The third criterion can be satisfied by using a stack of perfectly rectified PVC disks to make up the mobile internal cylinder to which the biofilm will attach itself. Using this apparatus, the growth of attached biomass can be followed in time by successive sampling of the disks, the ratio of volume

to surface area and the space time being kept constant by simple adjustment of the volume and input flow. The characteristics of the reactor, which was supplied with complex synthetic substrate (viandox, glucose, powdered milk, peptone, yeast extract and oligoelements), are shown in figure 2 and table 1. It should, however, be noted that, for aerobic biofilms, substrate removal was continuously checked by means of a TOD meter and the amount of dissolved oxygen (simultaneous supply of H_2O_2 and catalase) was controlled, the whole processes being supervised in real time by a micro-computer.

1-2. reactor
3. sampling
4. thermostat and mixer
5. temperature regulator
6. SS optical system
7. solenoid valve
8-10. pumps supplying H_2O_2 and catalase
11. pH meter
12. oxymeter
13. TOD meter
14-15. reservoir
16. analogue-digital converter
17. analogue-digital input-output interface
18. microcomputer (Apple II)

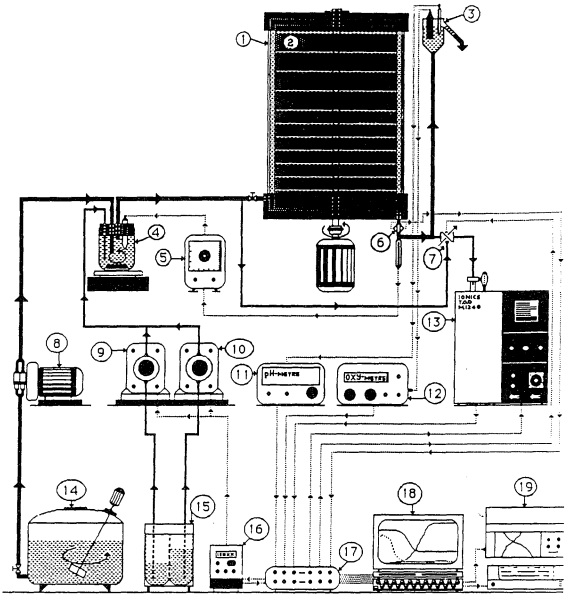


Figure 2. Diagram of aerobic reactor (Nguyen, 1989).

Table 1. Main characteristics of the reactor (Nguyen, 1989).

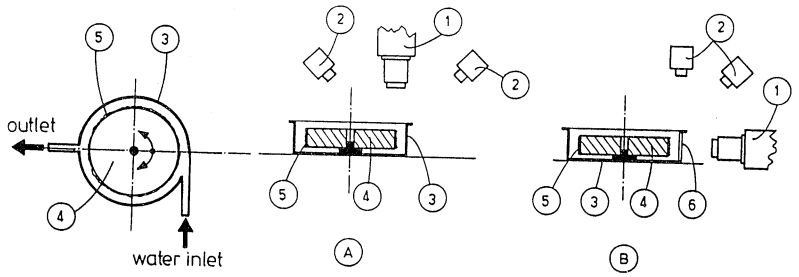
Description	Aerobic reactor
Reactor	
reacting volume (variable)	4.5-1.59 l
biofilm-liquid contact surface area (variable)	1760-565 cm2
Outer cylinder	
overall height	30.8 cm
inside height	25 cm
Inner cylinder (rectified PVC disk stack)	
number of disks used for sampling	7
disk diameter	20 cm
disk thickness	3 cm
Total height of stack	29.5 cm
Contact surface area per cm of disk height	62.8 cm2

2.2. CHOICE OF TECHNIQUES

2.2.1. *Analysis of physico-chemical parameters.*

- Biofilm thickness. It appears from the literature that the techniques for estimating this value are very diverse and sometimes ambiguous (Characklis et al., 1982 ; Huang et al., 1985). Moreover, the way the sample (biofilm) is conditioned can considerably alter the measurements.

To avoid these disadvantages, we developed a biofilm sampling, conditioning, and observation method based on the experimental arrangement shown diagrammatically in figure 3. This device, which has the same diameter as the cylinder, allows the biofilm to be kept in conditions very close to those prevailing in the reactor, thanks to the circulation of water. The biofilm's external structure can thus be correctly visualized, and its mean thickness and standard deviation deduced from statistical treatment of a large number of observations. The mean value of the thickness (δ_m) is estimated from photographic observations.



1. Wild stereoscopic microscope with micrometer objective
2. light sources
3. disk immersion dish
4. disk under observation, can be moved simply by rotation
5. biofilm
6. optical glass

Figure 3. Apparatus for biofilm characterization.

- Biofilm mass. Unlike most experimental arrangements put forward in the literature, our device provides easy access to this parameter. Biofilm is collected by simply scraping the sample disk, dried in the incubator, and weighed. Since the disk has a sufficient surface area, either one large fraction of the biofilm or several fractions can be recovered. A previous statistical study of the disks (each divided into four parts) allowed us to estimate the accuracy of the measurements, which depend in particular on how advanced growth is (30 % in latent phase for masses of the order of 50 $\mu g\ D.W.cm^{-2}$; 5 % in accumulation phase corresponding to masses of 100 $\mu g\ D.W.cm^{-2}$. In cases where greater accuracy was required, we carried out determinations of the products constituting the biofilm (proteins, polysaccharides) using the conventional methods of Lowry et al. (1951), and Dubois et al. (1956).

2.2.2. *Analysis of biological parameters.* We characterized biofilm activity using ATP measurement, following the protocol suggested by Cogny (1987), and dehydrogenase activity by Nguyen's method (1985).

3. Structural aspects of biofilms

3.1. OBSERVATION OF A BIOFILM

A biofilm is a set of micro-organisms and products which attaches itself to a solid support and increases in thickness until it forms a layer which is generally voluminous and jelly-like. But the external structure of the biofilm is not at all regular and uniform as many authors have assumed. Outgrowths appear in filaments, giving a very rough surface with irregularities. These observations allow us to judge the validity of the notion of characteristic thickness of a biofilm, i.e. to verify one of the hypotheses of the diffusional transport model.

Experiments performed on mixed, anaerobic (Belkhadir, 1986) and aerobic (Nguyen, 1989) biofilms have shown that film growth occurs in six distinct phases, as follows : latency, dynamic, linear, decrease, stabilization and detachment phases. We shall look at the kinetic characteristics of each phase in the next section.

Front- and side-view photographs were taken of the biofilm in each phase. The photographs of the growth of an anaerobic film are given in figure 4. We can see biofilm growth dynamics in which the induction phase corresponds to the establishment of micro-colonies on the support. These micro-colonies are initially evenly distributed over the support (photo A'), as stated by Christensen et al. (1988). After a relatively short time (10 hours), two phenomena are visible: the density of colonies on the surface has increased, as has the mass of individual colonies (photo B'). An increase in colony mass is thus observed without the support being completely colonized, even after 43 hours of culture time (photos C' and D').

A comparison of the front and side views of the biofilm brings to light an observation artefact connected with the techniques used. In the front view, the biofilm is seen to be discontinuous (colonies spaced out and uniformly distributed over the surface), whereas the side view gives the image of a continuous biofilm for which a characteristic thickness can be found. In fact, the side view juxtaposes colonies in the same plane of observation, which gives the impression of a pseudo-biofilm corresponding to the outer envelope of the colonies. In contrast to the hypotheses mentioned above, the biological films that we developed, did not lead to a plane uniform surface but, on the contrary, a relatively high heterogeneity of structures.

In all cases an initial stage was observed in which certain types of bacteria adhered to the surface (Capdeville and Nguyen, 1990), forming inoculation points from which the supports were colonized. This observation has been confirmed by other works (Hernandez et al., 1989) which consisted of a study of the preliminary stage of attachment of a mixed bacterial culture on special supports composed of synthetic polymers, such as ultrafiltration membranes, or thermoplastics (polyethylene, polypropylene, polystyrene, PVC). It is possible to change the surface properties of these supports, notably their hydrophobic character (hydrophilic-lipophilic balance) and the nature of the chemical functions and surface charges, by various techniques (cold plasma, coating, etc.). The stronger the positive surface charge of the support, the stronger this interaction proves to be (see figure 5). However, controlling the initial bacteria adherence conditions themselves remains difficult. Nevertheless, it is still possible to accelerate colonization of a support by slow growing bacteria, which is all the more justified when thermoplastics with non-porous surfaces are used.

All these results tend to show that the dynamics of colonization of a support does not involve successive superposition of layers of micro-organisms but rather obeys physical laws of interaction between the bacteria and the support (general law of occupation of an available area).

At macroscopic level, the surface structure of the aerobic biofilm is completely different from that of an anaerobic film from the linear growth phase onwards. If the dissolved oxygen content is greater than 1 mg O_2/l, the surface of the aerobic biofilm is very irregular because of the filamentous outgrowths (see figure 6), which give it a fairly large pseudo-thickness. The surface of the anaerobic biofilm, on the other hand, is more regular and it is not so thick. Thus, the reduction of the biofilm density is proportional to the increase in the oxygen concentration. These observations confirm those of Christensen et al. (1988) and Hamoda et al. (1987).

3.2. GROWTH KINETICS OF A MIXED BIOFILM

Our experiments on the growth of aerobic and anaerobic biofilms have enabled us to characterize the kinetics of these phenomena. It can be observed that the growth kinetics of the aerobic film (Nguyen, 1989) are identical to those of the anaerobic one (Belkhadir, 1989). For example, figure 7 shows the changes with time for substrate concentration (S) at the outlet of a continuous feed reactor, product concentration (if anaerobic), dissolved oxygen concentration (if aerobic), mass of fixed bacteria (M_b) and the resulting pseudo-thickness of biofilm. Biofilm growth can be described according to the following six phases:

3.2.1. Latency or activation phase. This corresponds to a phenomenon of adsorption of organic molecules and a phenomenon of fixation of isolated bacteria (Fletcher, 1980). We note the establishment of small, dispersed colonies of bacteria, preferentially localized at surface irregularities. The duration of this phenomenon depends enormously on the incoming substrate concentration and the surface properties of the support. This phase remains very difficult to control.

3.2.2. Acceleration or dynamic growth phase. This corresponds to very rapid development in the colonization of the support from the micro-colonies fixed initially. Since the organic substrate and oxygen are available in quantities largely exceeding consumption needs, the micro-organisms colonize the support with a maximum growth rate. In the side view, a thin layer characteristic of a bacterial film appears towards the end of this phase. During this phase we observe :

- a rapid increase in the rate of production of polysaccharides and proteins ;
- a rapid, massive fall in the substrate concentration ;
- a large oxygen consumption in the case of growth of an aerobic film. The initial dissolved oxygen content then becomes limiting and it becomes necessary to provide an outside source of oxygen (dissociation of H_2O_2 by an enzyme, for example) ;
- a great production of fermentation metabolites in the case of growth of an anaerobic film ;
- a very clear accumulation of fixed biomass.

In this phase we also observe that the substrate concentration at the outlet tends towards a minimum, non-zero limit value (S_{min}). This implies that the biofilm's capacity to remove substrate is tending to its maximum. In consequence, the changes in biofilm total dry matter (M_b) do not express these equilibrium states. To explain this phenomenon, Belkhadir (1986) suggests a new concept based on the existence of active and deactivated micro-organisms (M_a , M_d concept) making up the biofilm. The activated bacteria (M_a) are responsible for breaking down the substrate. They either constitute the departure point for new colonies or are situated at the edges of existing colonies. The inert or deactivated bacteria (M_d) no longer play any role in the depollution process. This biomass, normally situated inside the colonies is the result of the transformation of active bacteria which have lost their capacity to break down substrate but retain a certain enzymatic

activity. The deactivation, connected with an effect of confinement due to the accumulation of new cells, must depend on the fraction of the surface area occupied. Belkhadir (1986) puts forward the diagram shown in figure 8.

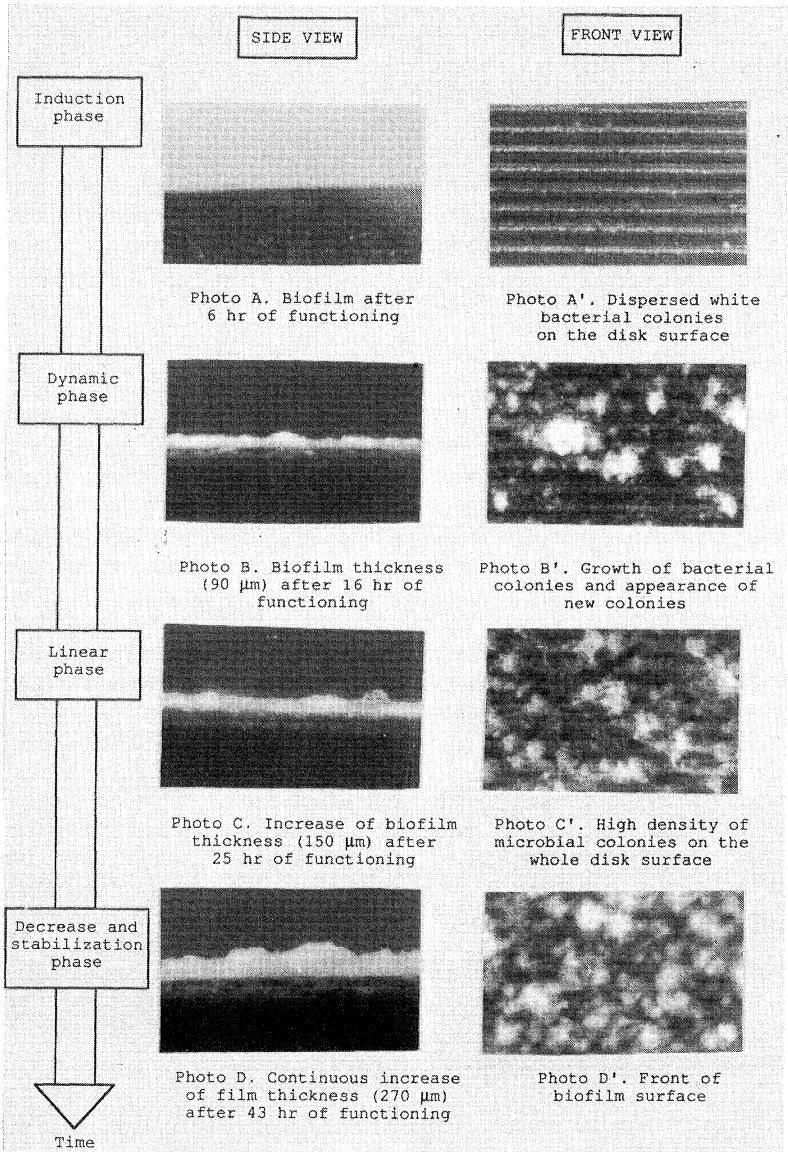


Figure 4. Microscopic observation (x225) of an aerobic biofilm growth (Nguyen, 1989).

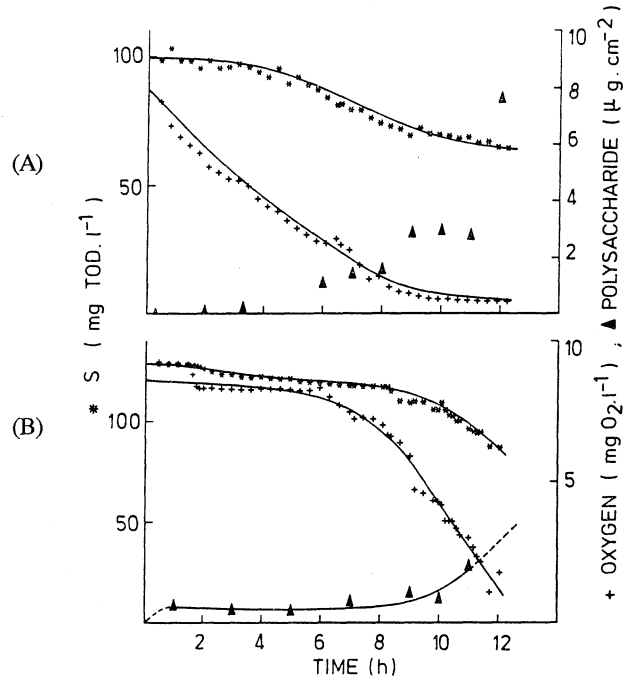
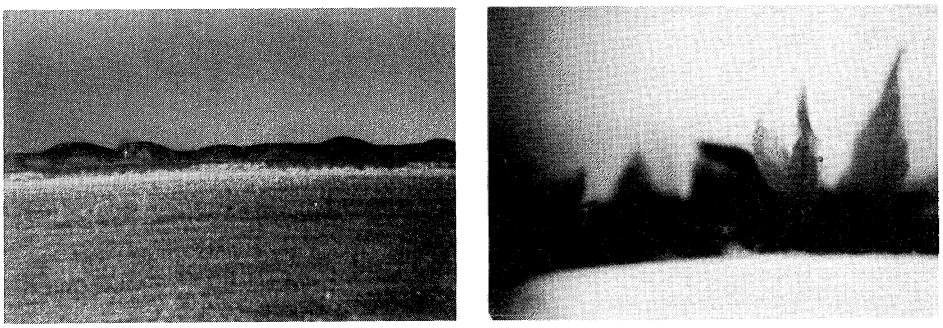


Figure 5. Interaction of a mixed population of heterotrophes with anionic UF membrane (IRIS 3050RP) : (A) free membrane, (B) membrane coated with sodium oleate (Hernandez et al., 1989).



(A) (B)

Figure 6. Side view (x130) of (A) anaerobic biofilm and (B) aerobic biofilm in the stabilization phase (Belkhadir, 1986).

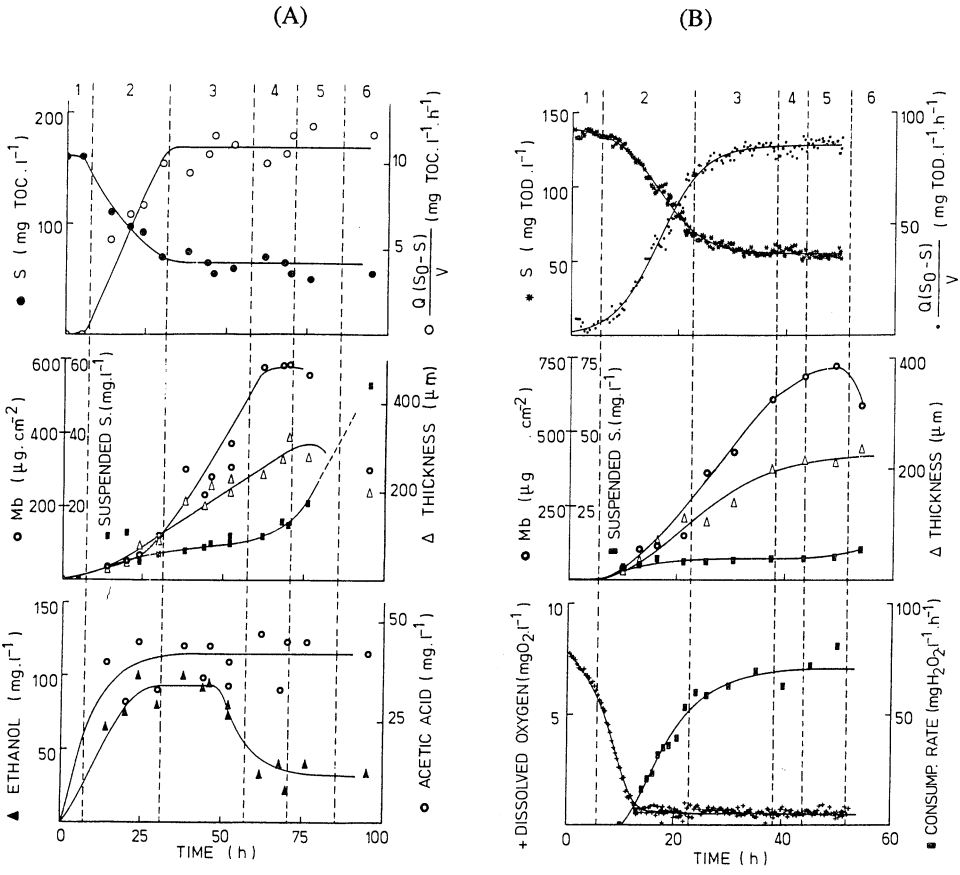


Figure 7. Growth kinetics of (A) an anaerobic biofilm (Belkhadir, 1986) and (B) an aerobic biofilm (Nguyen, 1989).

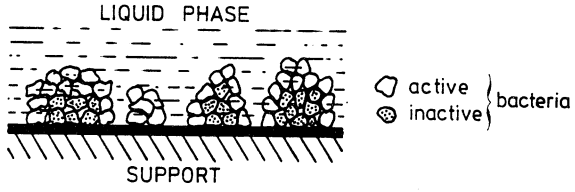


Figure 8. Diagram of bacterial colony development on a support (Belkhadir, 1986).

As far as the depollution process is concerned, the acceleration phase plays an important role. It corresponds to the setting up of a permanent mode of functioning with respect to the active bacteria and thus with respect to the substrate consumed. It is for this reason that the phase is known as the dynamic growth phase.

3.2.3. Linear accumulation phase. This corresponds to the accumulation of biomass on the support at a constant rate. Its characteristics are :

- a constant substrate concentration at the reactor outlet, expressing a permanent mode of functioning in the liquid phase ;
- a constant oxygen consumption in the case of aerobic biofilm growth ;
- constant product concentrations in the case of anaerobic biofilm growth.

From the macroscopic standpoint, the side view of a biofilm shows a uniform thickness from the linear accumulation phase onwards. The plan view shows that the colonization of the support is completly realized. However, the aerobic biofilm surface structure may be very irregular because of the presence of filamentous outgrowths depending on the dissolved oxygen concentration.

From the (M_a, M_d) concept viewpoint, the linear development of total biomass (M_b) with time indicates a slowing of the process relative to the dynamic growth phase. The causes of this, which are connected with support saturation, are multiple and concern inhibition effects due to cell density or the accumulation of toxins. This conclusion is the same as that drawn by Christensen et al. (1988) who hypothesize that the products in the micro-environment of the bacteria and the secondary products inside the biofilm may create environmental conditions unfavourable to bacterial activity. According to Belkhadir (1986), these effects can be grouped together under the term "confinement".

To explain the inhibiting effects of the products, we must divide them into three types :

- primary products : these are the metabolites released into the liquid phase during growth (case of anaerobic processes where the fermentation products are ethanol, acetic acid, etc.) ;
- secondary products : these are extra-cellular metabolites remaining adsorbed on the membrane (e.g. exopolymers) ;
- tertiary products : these are intra-cellular metabolites, transformed and released during cell lysis (metabolites re-released).

These products can have two different actions :

. like the stacking up of layers of bacteria, they can modify the transport conditions in the neighbourhood of the cell, with a stifling or masking effect. This role can probably be attributed to the secondary products ;

. they can have a toxic effect in the physiological sense of the term. This role may particularly concern the primary products and the metabolites that are re-released.

From a practical point of view, the linear accumulation phase presents little interest for depollution, given that the maximum substrate degradation potentials have already been reached. In contrast, it favours a modification of the volume properties of the bioparticles in a fluidized bed.

3.2.4. Decrease phase. This corresponds to the transition between the accumulation of biofilm at a constant rate and its stabilization at maximum mass and thickness values. It is expressed by a slowing down of growth connected with hydrodynamic stresses which prevent the accumulation of new cells. During this phase, physical phenomena become preponderant relative to biological

ones. The biofilm becomes sensitive above all to shear stresses (Rittman, 1982), in particular in the case of aerobic biofilms obtained with a high concentration of dissolved oxygen (mainly because of their very loose, filamentous structure). This phenomenon prevents any additional accumulation of biofilm. It should be noted that, during this phase, the amount of suspended solids in the effluent at the outlet of the reactor increases all the time. To justify that these suspended solids are made up of biofilm detached by the shearing forces, Belkhadir (1986) has shown, through observations under the microscope, that the internal structure of the fixed biofilm and that of the suspended material are practically identical. This phenomenon is all the more marked in aerobic biofilms when the dissolved oxygen concentration is high. This shows that a filamentous structure is very sensitive to shearing forces.

At the end of this phase, the biofilm's total mass and thickness tend towards maximum values ; a steady state is established for the biofilm.

3.2.5. Stationary or biofilm stabilization phase. This corresponds to a balance between the loss of biomass due to physical stresses and the growth of new cells at the edge of the biofilm. Its characteristics are constant maximum values for the biofilm mass and thickness, which corresponds to the establishment of a steady state for the biofilm, the steady state having already been reached in the liquid phase. This phase is generally short-lived and depends on certain running conditions such as the substrate supply concentration and the shearing forces. In certain cases (high substrate concentration, high rotational speed) this phase does not seem to exist.

3.2.6. Detachment phase. The detachment of the biofilm is a random phenomenon depending on the behaviour of the bacteria fixed directly on the support. Too great an accumulation of biofilm results in this phase being characterized by biological factors like cell lysis in the deep layers and changes in the interactions between the bacteria and the support, and physical factors like the action of the force of gravity and tangential forces on the mass of the biofilm. In this phase, the suspended solids in the liquid increase and their permanent functioning relative to the substrate and biomass concentrations are out of balance. Moreover, an increase in the amount of the substrate in the liquid phase is observed, in connection with a partial or total loss of the biofilm. A new biofilm starts to grow in the areas where the old film has detached itself. This phase generally marks the end of biofilm increase.

3.2.7. Conclusion. The most important consequence of the biofilm growth study is to show that two steady states are established :

- a steady state functioning established in the liquid phase, which is reached at the end of the dynamic phase and has corresponding very thin observed biofilm pseudo-thicknesses (50 μm at most) ;
- a steady state functioning with respect to the observed biofilm mass, which is reached in the stabilization phase and corresponds to thicknesses of a few hundred microns.

Figure 9 shows all the phases with their kinetic and structural signification.

Comment : In the case of a biofilm, ATP, which is a characteristic of living cells, should reflect the metabolic activity of the active micro-organisms only. But Nguyen (1989) indicates that the ATP, protein and polysaccharide parameters are functions of the type $y = a.x$ with respect to the total mass of the biofilm (M_b). This implies that the quantity of active biomass in the biofilm increases proportionally to the total mass of the film, which should be expressed by a decrease in

substrate concentration in the fermentor beyond the dynamic growth phase. This is not confirmed by experiment. So it seems that this quantity is not simply characteristic of substrate metabolizing activities by the active micro-organisms. The intrinsic substrate removal rate thus remains the essential characteristic showing the potential of the active bacteria.

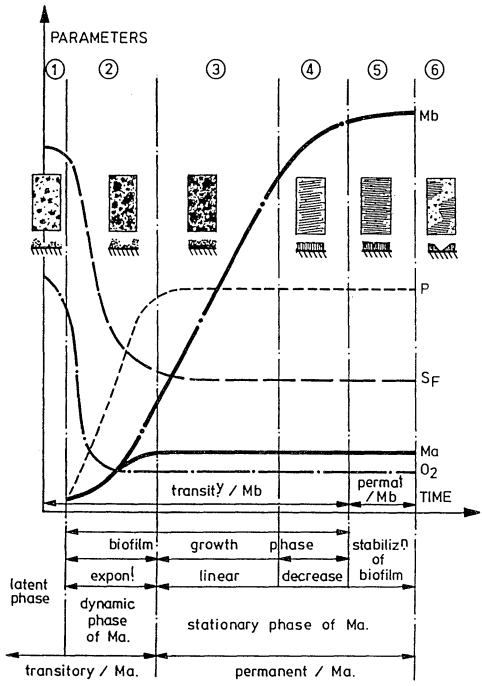


Figure 9. Presentation of different growth phases of biological film (Nguyen, 1989).

4. New foundations for the modelling of biofilms

4.1. THE KINETICS OF BIOFILM GROWTH

The modelisation of biomass growth is based on the concepts of active micro-organisms (M_a) and deactivated micro-organisms (M_d). The active bacteria are responsible for the breaking down of the substrate and are characterized by a specific rate of growth (μ_o). The deactivated bacteria play no role in the substrate breakdown process, although they can help to maintain certain enzyme activities. Using this definition, the total biomass (M_b) is defined as :

$$M_b = M_a + M_d \qquad (1)$$

Various models are generally used for modelling the kinetics of growth. Their starting point is a first order differential equation relating to the concentration of micro-organisms which can be written as follows :

$$dX/dt = \mu.X \quad \text{or} \quad dX/dt = \mu.X - k.I.X$$

where : μ : the rate of growth
k : the coefficient of arrested growth or mortality
X : the concentration of the biomass
I : the concentration of inhibitory product.

The objective of these models is to define the rate of growth, and they represent the hypothesis that, at any given moment, the population of bacteria in a culture will multiply as long as either some substrate exists or the concentration of inhibitory product is not critical ; the speed of multiplication must be continually modified.

To describe the kinetics of the fundamental growth phases of a biofilm, Belkhadir et al. (1988) proposed :

- the intrinsic kinetics of growth of the active biomass (r_{Ma}) of order 0 in relation to the substrate and order 1 in relation to the active bacteria :

$$r_{Ma} = \mu_o . M_a \qquad (2)$$

where : μ_o : the maximum growth rate (T^{-1})
M_a : the active mass per unit of surface area (ML^{-2}).

- the kinetics of deactivation (r_{Md}) dependant on the cell density (the confinement effect) and the accumulation of inhibitors :

$$r_{Md} = k_1.I.M_a \qquad (3)$$

where : k_1 : the constant of deactivation ($M^{-1}L^3T^{-1}$)
I : the concentration of inhibitors (ML^{-3}).

The rate of accumulation of active bacteria on the support is given by :

$$(dM_a/dt)_{acc} = r_{Ma} - r_{Md} \qquad (4)$$

Furthermore, it is stated that the concentration of inhibitors is proportional to the cell density, and that at the end of the dynamic growth phase the quantity of active biomass is maximal and constant $(M_a)_{max}$, since the concentration of substrate at the reactor outlet is constant and minimal. From which we get :

$$(dM_a/dt)_{acc} = \mu_o.M_a - k_2.M_a^2 \qquad \text{where :} \quad k_2 = \mu_o/(M_a)_{max}$$

From here the following differential equations are deduced :

$$(dM_a/dt)_{acc} = \mu_o.M_a.[1 - M_a/(M_a)_{max}] \qquad (5)$$

and : $$r_{Md} = (dM_d/dt)_{acc} = \mu_o.M_a^2/(M_a)_{max} \qquad (6)$$

The expression for the rate of accumulation of active micro-organisms in the reactor results in a sigmoid model based on a mathematical function known as a logistic function. This expression, which gives sigmoid shapes like those observed in autocatalytic phenomena had considerable doubt cast upon it by Monod (1942). But in a study performed in our laboratory on the growth of dispersed cultures from the analysis of gaseous metabolites by mass spectrometry, Chang (1988) confirms this concept, bringing in, however, the idea of viable and non-viable bacteria.

Furthermore, the experimental observations of Belkhadir (1986) show that the density of micro-colonies depends on the surface area available on the support. The fraction of the surface

covered (a/A_o) is therefore introduced into the kinetic expression, equation (5), bearing in mind that M_a and $(M_a)_{max}$ are proportional, respectively, to the biofilm liquid exchange surface area corresponding to the exchange surface area at the instant t, and to the total exchange surface area at the end of the dynamic phase. We thus arrive at :

$$(dM_a/dt)_{acc} = \mu_o.M_a.B.(A_o - a)/A_o \qquad (7)$$

where : a : the surface area covered by the micro-colonies at the instant t

A_o : the initial surface area of the support

B : a correction term for the difference between the surface area of the model and that of the physical reality of the phenomenon.

This equation shows that as long as there is surface area available there will be increase in the quantity of active biomass (biological space concept).

The speed of accumulation of the active bacteria, in its differential form, equation (5), is a Riccati equation, easily integrated in certain conditions :

$$M_a \text{ in }]o, (M_a)_{max}[\text{ and } \mu_o \neq 0$$

Integration of equations (6) and (5) gives, respectively :

- the variation of the mass of active bacteria (M_a) with time

$$M_a = \frac{(M_a)_o.e^{\mu o t}}{1 - [(M_a)_o/(M_a)_{max}].[1-e^{\mu o t}]} \qquad (8)$$

where $(M_a)_o$ represents the initial active mass corresponding to the start of the dynamic phase of growth, which is also the time origin.

- the variation of the mass of deactivated bacteria (M_d) with time

$$M_d = (M_a)_{max}.Ln\,[1-(M_a/(M_a)_{max}).(1-e^{\mu o t})] + \frac{(M_a)_o.(1-e^{\mu o t})}{1 - [(M_a)_o/(M_a)_{max}].[1-e^{\mu o t}]} \qquad (9)$$

- and the variation of the observed mass of the biofilm (M_b) with time, knowing that M_b is equal to the sum of M_a and M_d

$$M_b = (M_a)_{max}.Ln[1-(M_a/(M_a)_{max}).(1-e^{\mu o t})] + \frac{(M_a)_o}{1 - [(M_a)_o/(M_a)_{max}].(1-e^{\mu o t})} \qquad (10)$$

It should be noted that these expressions give the variation of the different masses, assuming that biomass loss by hydraulic shearing remains negligible in the first part of the process. From this concept, it is possibe to simulate the variations with time of the active biomass, of the deactivated biomass, and of the total mass of the biofilm, in the dynamic and linear growth phases of a biofilm.

If we take relatively short time, corresponding in fact to the dynamic phase, expressions (8) and (10) can be simplified to :

$$M_a = (M_a)_o.e^{\mu o t} \qquad \text{and} \qquad dM_b/dt \# \mu_o.M_b \qquad (11)(12)$$

This expresses a logarithmic variation of biofilm mass such as reported in the literature by La Motta (1976), Trulear et al. (1982), and Characklis et al. (1982).

Under these conditions, the maximum growth rate (μ_o) can be estimated in the semi-logarithmic plane [Ln(M_b), time] (Belkhadir, 1988). However, this estimate may be inaccurate because of the few experimental points obtained in the dynamic phase, the duration of which depends notably on the feed substrate concentration. A better estimate of this parameter can be

made by parameter adjustment using Hooke and Jeeves method in the plane [S, time] (Nguyen, 1989).

As far as the (M_a, M_d) concept is concerned, the linear variation in the mass of dry matter in the biofilm with time shows a slowing down of the growth process relative to the dynamic phase. Under these conditions, i.e. if the time is sufficiently long, equations (9) and (10) can be simplified to give the following expressions :

$$\bullet M_d = \mu_o (M_a)_{max}.t + (M_a)_{max}.[Ln\,[(M_a)_o/(M_a)_{max}] - 1] \quad (13)$$

then $$M_d = K.t + C_1 \quad (14)$$

$$\bullet M_b = \mu_o.(M_a)_{max}.t + (M_a)_{max}.[Ln\,[(M_a)_o/(Ma)_{max}] + 1] \quad (15)$$

then $$M_b = K.t + C_2 \quad (16)$$

with $$K = \mu_o.(M_a)_{max} \quad (17)$$

which represents a constant rate of biofilm accumulation on the support.

then $$r_{Ma} = r_{Md} = K = \mu_o.(M_a)_{max} \quad (18)$$

Equation (18) shows that there is a balance between the quantity of active bacteria and the quantity of deactivated bacteria. The latter accumulate on the support at a constant rate and are responsible for the observed accumulation of biofilm. The constant K can thus be determined in the plane [M_b, time] (Belkhadir, 1988). Moreover, since the maximum growth rate (μ_o) can be determined from the dynamic phase, the maximum quantity of attached active bacteria $(M_a)_{max}$ can be deduced from expression (17).

Under these conditions, the model can be adjusted and simulations performed for the dynamic and linear phases. Figure 10 shows a good fit between the experimental points and the theoretical model, indicating that the hypotheses of the (M_a, M_d) concept are coherent. This would confirm that the biological process is controlled by physiological factors such as inhibition and diffusion limitations in the vicinity of the bacteria.

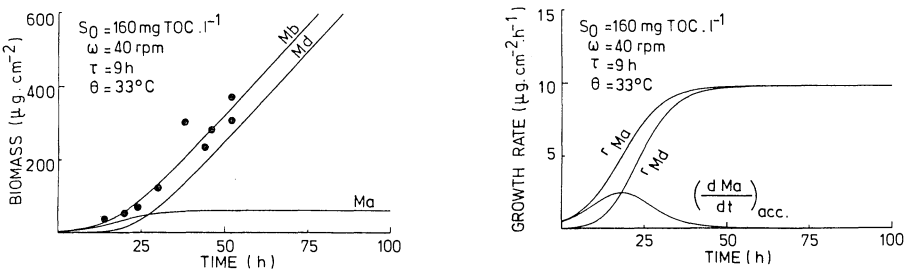


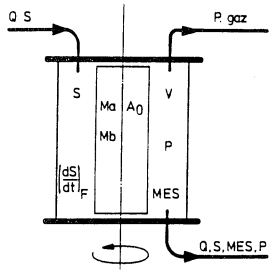
Figure 10. Adjustement and variation with time of M_a, M_d and M_b for an anaerobic biofilm (Belkhadir, 1986).

4.2. THE KINETICS OF SUBSTRATE REMOVAL

In dealing with the difficulty of quantifying the active biomass of a biofilm by ATP measurements, it is necessary to control the kinetics of the breaking down of the substrate, in order to be able to interpret the kinetics of biofilm growth. Our experimental results permit us to study an original model of the removal of substrate, based on the following hypotheses :

- according to the (M_a, M_d) concept, only the bacteria situated in the peripherical layer of the biofilm are responsible for the breaking down of the substrate ;
- the influence of substances which are in suspension at the time of the dynamic and linear phases is relatively weak, and the fraction of the substrate consumed by these is negligible compared with that used up by the fixed bacteria. Maintenance phenomena are also ignored.

The substrate balance in transient mode at the input and output of a completely mixed fermentor can be written :



(internal variation) = Input - Output - Consumption

$$V.(dS/dt)_F = Q.(S_0 - S) - V.(r_S)_G \quad (19)$$

$$\text{thus } (dS/dt)_F = D.(S_0 - S) + (r_S)_G \quad (20)$$

with

$(r_S)_G$ = overall substrate removal rate ($ML^{-3}T^{-1}$)
V = reactor volume (L^3)
Q = feed flow rate (L^3T^{-1})
Q/V = D = dilution rate (T^{-1})

If we neglect substrate consumption by matter in suspension, and consider a global conversion yield (Y_G) taking into account maintenance and metabolite production reactions, the global substrate removal rate can be written in the form :

$$(r_S)_G = - \frac{A_0}{V.Y_G} .\mu_0.M_a \quad (21)$$

By introducing equation (21) into expression (20) we obtain :

$$(\frac{dS}{dt})_F = (\frac{d(S_0 - S)}{dt})_F + D.(S_0 - S) = \frac{A_0}{V.Y_G} . \mu_0 .M_a \quad (22)$$

In the steady state in the liquid phase, characterized by a minimum substrate concentration (S_{min}) at the fermentor outlet and a maximum quantity of active biomass $(M_a)_{max}$, we can write :

$$S_0 - S_{min} = \frac{A_0}{V.Y_G.D} .\mu_0.(M_a)_{max} \quad (23)$$

thus

$$Y_G = \frac{A_0 .\mu_0 .(M_a)_{max}}{Q.(S_0 - S_{min})} = \frac{K. A_0}{Q.(S_0 - S_{min})} \quad (24)$$

The general equation for substrate removal in transient mode is obtained by replacing the value of Y_G (24) in (22) :

$$(\frac{d (S_0 - S)}{dt})_F + D.(S_0 - S) = D.(S_0 - S_{min}). \frac{M_a}{(M_a)_{max}} \quad (25)$$

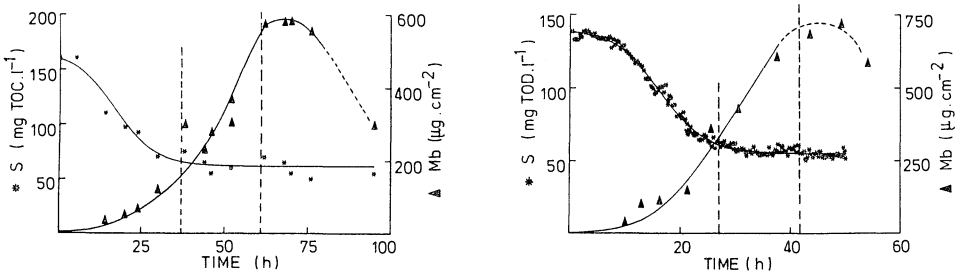
where M_a is a function of time given by equation (8).

Two cases can be distinguished for solving equation (25) depending on whether internal variation is negligible or not. In the case where the internal variation is negligible compared with

the term (S_0 - S), expression (25) associated with equation (8) gives, directly, the substrate concentration variation at the fermentor outlet as a function of time :

$$S = S_0 - \frac{S_0 - S_{min}}{(M_a)_{max}} \cdot \frac{(M_a)_0 \cdot e^{\mu_0 t}}{1 - [(M_a)_0/(M_a)_{max}].[1 - e^{\mu_0 t}]} \qquad (26)$$

Figure 12 shows example of the fit of the curves of (S) and (M_b) as a function of time for anaerobic and aerobic biofilms.



(A) (B)

Figure 11. Growth and substrate removal kinetics for an (A) anaerobic biofilm (Belkhadir, 1986) and (B) aerobic biofilm (Nguyen, 1989).

It is interesting to characterize the rate per unit volume of the substrate removal reaction (k_{ov}) expressed in mg of substrate consumed per unit volume of active biofilm per unit time ($ML^{-3}T^{-1}$).

From equation (21), and expressing (M_a) as a function of the density of active cells per unit volume of active biofilm (X_a) and of an active pseudothickness (δ_a) defined relative to the geometrical surface area, we obtain :

$$A_0.M_a = X_a.\delta_a.A_0 \qquad (27)$$

The global reaction rate can be expressed as :

$$(r_s)_G = -\frac{\mu_0.X_a}{Y_G} \cdot \frac{A_0}{V} \cdot \delta_a \qquad (28)$$

thus

$$k_{ov} = \frac{\mu_0.X_a}{Y_G} \qquad (29)$$

By definition, the flow of substrate consumed in the biofilm is equal to the speed of the substrate removal reaction, expressed with respect to the surface area of the biofilm, taken to be A_0 : $F_G = k_{ov} \cdot \delta_a$ (30)

For a steady state in the liquid phase, we have :

$$(F_G)_{max} = k_{ov} \cdot (\delta_a)_{max} = Q.(S_0 - S_{min}) / A_0 \qquad (31)$$

By combining equations (29) and (34), we obtain the equation which expresses the changes in

k_{ov} with S_o :

$$k_{ov} = (k_{ov})_m \cdot S_o/(S_o + S_{oc}) \quad (32)$$

where $(k_{ov})_m = \mu_{om} \cdot X_A/Y_G$

Similarly, by combining equations (30) and (32), we have:

$$F_G = (F_G)_m \cdot S_o/(S_o + S_{oc}) \quad (33)$$

where $(F_G)_m = (k_{ov})_m \cdot (\delta_a)_{max}$

Using the maximum values for flow and k_{ov} it is possible to deduce the maximum active pseudo-thickness $(\delta_a)_{max}$. In our studies on anaerobic and aerobic biofilms, $(\delta_a)_{max}$ has always been less than 60 µm. However, this pseudo-thickness represents a great overestimation, given that the actual surface area of the biofilm is much larger than the geometrical surface area (particularly in the case of aerobic biofilms).

5. Study of the various factors influencing biofilm growth

After many observations in the study of the various factors influencing biofilm growth and substrate removal kinetics, it appears that the influence of the initial substrate concentration (S_o) is often debatable (Ohgaki et al., 1978 ; Molin et al., 1982 ; Characklis et al., 1988). Kornegay et al. (1968), Ohgaki et al. (1987), and Rittman et al. (1981) consider that the maximum growth rate (μ_{om}) and the threshold substrate concentration (K_s) are constants independent of the supply concentration (S_o). On the other hand, La Motta (1976) noted a clear dependence of μ_{om} on S_o, which can be expressed by the equation put forward by Gaudy et al. (1973) :

$$\mu_o = \mu_{om} \cdot S_o / (S_o + S_{oc}) \quad (34)$$

where μ_o = exponential growth rate (h^{-1})
μ_{om} = maximum exponential growth rate (h^{-1})
S_{oc} = initial substrate concentration for which $\mu_o = \mu_{om}/2$.

Moreover, this model has been experimentally confirmed by Belkhadir (1986) in a fundamental study of an anaerobic biofilm and also by Nguyen (1989) for a fixed aerobic culture in a ring reactor. These authors brought to light the main factors influencing biofilm growth and substrate removal kinetics (for a substrate of a known, constant type), such as initial substrate concentration, dissolved oxygen concentration, and physical parameters like hydraulic residence time and shearing force (which in this case was connected with the speed of rotation of the disks).

5.1. INFLUENCE OF THE CARBON SUBSTRATE CONCENTRATION

The changes in quantity of aerobic biofilm (M_b) in the dynamic and linear phases after parametric adjustment show that the greater the S_o concentration, the more dynamic the growth (figure 12).

We thus have a relationship linking the exponential growth rate (μ_o) and the biofilm accumulation rate in the linear phase (K) depending on S_o (figure 13). Furthermore, the overall substrate removal rate $(r_s)_G$ is controlled by the S_o concentration.

Consequently, the substrate removal rate per unit volume (k_{ov}) expressed in mg of substrate

consumed per unit volume of active biofilm per unit time is not constant but depends on S_0 (figure 14). The maximum active masses of fixed micro-organisms $(M_a)_{max}$ are constant whatever the S_0 concentrations (figure 15). The same results have been observed with anaerobic biofilm (Belkhadir, 1986).

Our results show a surface reaction which is independant of the biofilm thickness (up to 200 to 300 μm) and the reaction rate is not constant but depends, in particular, on the concentration at the reactor inlet. This indicates that the substrate removal remains reactive.

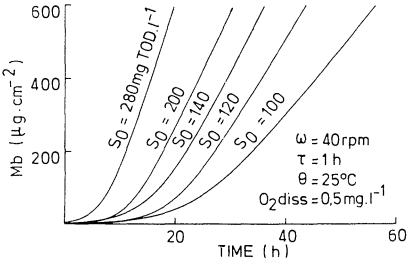


Figure 12. Influence of S_0 on M_b for an aerobic biofilm (Nguyen, 1989).

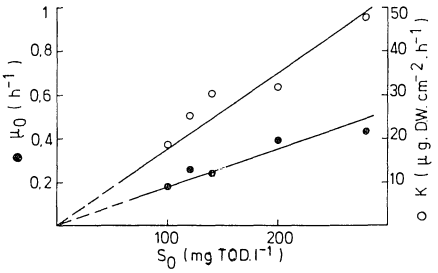


Figure 13. Variations of μ_0 and K with S_0 for an aerobic biofilm (Nguyen, 1989).

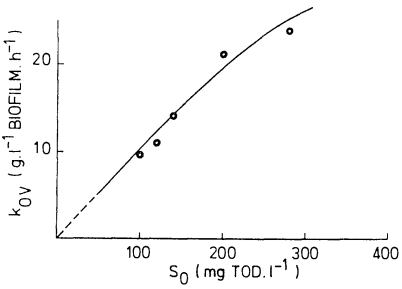


Figure 14. Influence of S_0 on k_{ov} for an aerobic biofilm (Nguyen, 1989).

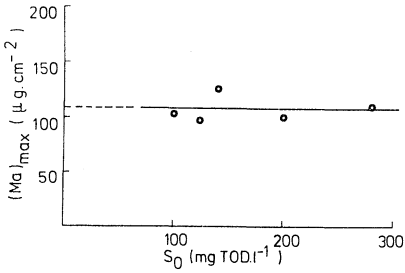


Figure 15. Variation of $(M_a)_{max}$ with S_0 for an aerobic biofilm (Nguyen, 1989).

5.2. INFLUENCE OF THE DISSOLVED OXYGEN CONCENTRATION

For attached aerobic cultures, Nguyen (1989) has performed a series of experiments in which the dissolved oxygen concentration was kept constant in the reactor by supplying H_2O_2 and catalase. The results obtained show that the exponential growth rate of the biofilm increased rapidly with the dissolved oxygen content, becoming constant above a value of 1 mgO_2/l (figure 16). The accumulation rate, on the other hand, shows a maximum for this oxygen concentration. This is the consequence of a change in the structure of the biofilm and the micro-organisms composing it. At low dissolved oxygen concentrations ($\leq$ 2 mgO_2/l) the biofilm has a dense structure of non-filamentous bacteria on which shear stresses have little effect. With rising dissolved oxygen concentrations, the structure becomes hairy and homogeneous with filamentous bacteria that are very sensitive to hydrodynamic forces.

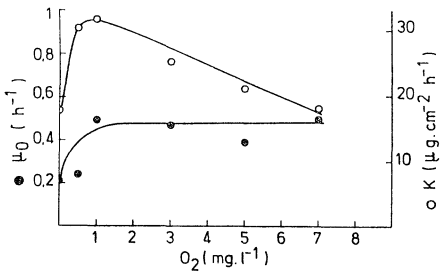


Figure 16. Variation of biofilm growth and accumulation rates depending on dissolved oxygen concentration (Nguyen, 1989).

5.3. INFLUENCE OF SHEAR STRESSES

The shear and frictional forces are linked together and have an influence on the colonisation of a granular support, particularly in a three-phase fluidized bed. Where frictional forces are

concerned, it is also necessary to take account of the quantity of support material in the reactor (Heim da Costa, 1989). For fixed anaerobic cultures, Belkhadir (1986) has shown that the influence of factors such as hydraulic residence time and speed of rotation on the kinetic constants $\{\mu_o$, K and thus $(M_a)_{max}\}$ is slight. In contrast, the mass and thickness of the biofilm are sensitive to the speed of rotation and remain maximum at a critical value of 40 rpm, which corresponds to a tangential speed of 48.2 cm/s. In the case of a three-phase fluidized bed, Lertpocasombut (1991) has shown that the total mass of biofilm depends on the hydrodynamics imposed by the surface speed of the gas (see figure 1).

6. Biofilm modelling : comparison of the two concepts

The molecular diffusion of an organic compound through a biofilm is a theoretical concept accepted because of its similarity to heterogeneous chemical catalysis (Bird, 1960). The resulting biofilm model is based on the following hypotheses :

- plane, homogeneous film ;
- permanent state of the biofilm (characterized by a constant thickness) ;
- substrate transport in a single direction by molecular diffusion ;
- intrinsic zero-order kinetics with respect to the substrate : $r_s = k_{ov}$ = constant.

The flow of substrate consumed in the thin biofilm is equal to the substrate removal rate, expressed with respect to the biofilm surface area, which is taken to be equal to the geometrical surface area A_o :

$$F_G = k_{ov} . \delta_a$$

where δ_a represents the thickness of film penetrated by the substrate.

For a thick biofilm, we introduce the penetration rate $\beta = (\delta_a)_{max}/e$, which represents the active fraction of the biofilm relative to its total thickness (e).

$$F_G = k_{ov} . \beta . e$$

Based on this concept, the following conclusions are to be found in the literature :

- in a thin biofilm ($\beta \geq 1$), the diffusion rate is high relative to the reaction speed and the mode of functioning is said to be reactional ;
- in a thick biofilm ($\beta < 1$), the diffusion rate is low relative to the reaction speed and the mode of functioning is said to be diffusional.

Thus, if for a thick (partially penetrated) biofilm the limiting factor is transport by diffusion, the film can become completely active (totally penetrated) for a limit substrate concentration (S_l) at the interface such that :

$$\delta_a = e = (2\, De/k_{ov})^{1/2} . S_l^{1/2} \qquad (\beta = 1).$$

According to Harremoës (1977), a 200 µm thick biofilm becomes totally active with a concentration of 1300 mg glucose /l. This supposes that the micro-organisms in the zone that is inactive at low substrate concentrations keep the same growth potential as those in the active zone. In other words, there are no growth limiting factors (inhibition, influence of cell density, products elaborated, etc.) in a thick biofilm. Moreover, since the flow at the film-liquid interface is proportional to the active thickness (δ_a) and as k_{ov} is taken to be constant, if the substrate concentration at the reactor inlet (S_o) increases, the concentration at the interface increases, which necessarily leads to an increase in the active thickness (δ_a), and thus in the active mass (M_a).

But the experiments carried out by La Motta (1976), Belkhadir (1986) and Nguyen (1989)

show that a) k_{ov} is not constant (dependent on S_o) and b) that M_a and thus δ_a, hardly change when the liquid phase is in a steady state (whatever the value of S_o). This shows that the mode of functioning remains reactional whatever the thickness of the biofilm. The substrate may completely penetrate the biofilm but the micro-organisms are incapable of metabolizing it.

It is thus clear that the diffusion-reaction concept does not take account of the biological phenomena and that it underestimates the reactivity (k_{ov}) of the film by overestimating the active thickness (δ_a). The (M_a, M_d) concept better describes the maturing and reactivity of a biofilm. The resulting active thicknesses are of the order of a few tens of μm if the geometrical surface area A_o of the film is used as a basis. Given the heterogeneous nature of the surface, this, too, is a considerable overestimate. It thus seems that, with respect to fixed culture treatment processes for urban wastewater, it is not necessary to encourage a biofilm to grow thicker but, rather, to favour the use of thin films.

7. Thin film fixed culture processes

The three-phase fluidized bed is currently one of very few processes capable of producing thin biofilms. However, small particle beds (sand, etc.) are limited particularly by instability of the bed (bioparticle washout) and by an insufficient transfer of oxygen in the liquid phase.

As a result of the fundamental research carried out in our institute on the kinetics of fixed bacteria growth (Belkhadir, 1986 ; Nguyen, 1989), new granular materials have been developed which enable us to resolve the above mentioned problems. This support, known as O.S.B.G. (Optimized Support for Biological Growth) is composed of composite plastic balls which have undergone surface oxidation to render them hydrophilic (Capdeville et al., 1988). The materials have optimized volume (diameter, density, shape) and surface (critical surface tension for wetting, surface charges, surface energy) properties.

Laboratory tests are currently being performed with the help of ANVAR (the French national agency for the valorization of research) and the Lyonnaise des Eaux company to put these materials to use in turbulent and stirred fluidized reactors and also in submerged filtration reactors.

The research work being done at present by Hatzifotiadou et al. (1988, 1989) on hydrodynamics and transfer of matter in three-phase fluidized beds shows in particular that the overall oxygen transfer rate (k_La) is hardly affected by the presence of O.S.B.G. as compared with conventional materials (sand, clay, pummice stone, etc.).

Investigations have been carried out using O.S.B.G. materials in order to control the biological aspects of three-phase fluidized bed reactors, used to eliminate carbonaceous pollutants from synthetic effluent (Lertpocasombut, 1991). The biofilm growth brings about two permanent systems of operation : the first system is established very rapidly during the liquid phase when the concentration of organic substrate becomes constant ; the second system, concerning the quantity of fixed biomass on the support, establishes itself much more slowly after a period of accumulation (see figure 17). In these experimental conditions the active pseudo thickness $(\delta_a)_{max}$, calculated in relation to the geometrical surface of the biofilm (overestimation), varies between 15 and 35 μm.

The bacterial activity of the biofilm is measured by the dehydrogenase activity, using the I.N.T. method (Nguyen, 1985). This permits us to determine the global metabolic activity of the fixed bacteria (see figure 1). By use of the I.N.T. parameter, we see firstly the establishment of the two permanent systems in the dynamic phase and during the accumulation of biofilm growth, and secondly the effect of an increase in the shearing forces (by increasing the flow of air) on the

specific activity of the biofilm.

In creating a thin biofilm, the three-phase fluidized bed permits the elimination of from 10 to 15 kgTOD/m^3.d, leading to a total dry matter concentration in the reactor in the order of 0.5 g/l. These conditions allow very stable reactor operation to be obtained (no bioparticle drag) and a particularly low amount of sludge to be produced (0.1 to 0.2 kgD.W. produced per kg soluble TOD eliminated), which can be explained by the very high catabolic activity of the biofilm.

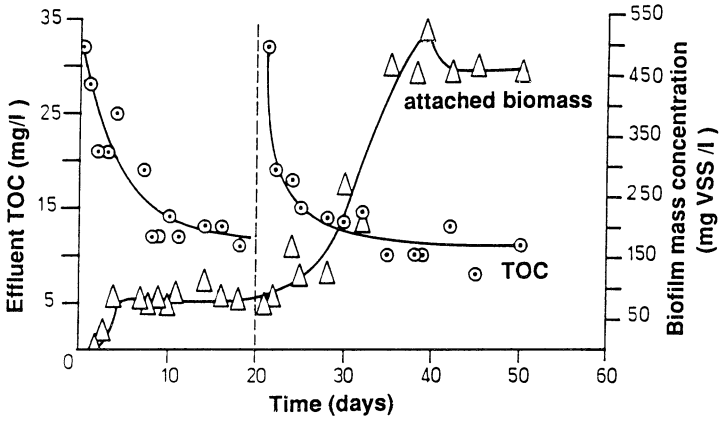


Figure 17. The dynamics of biofilm growth in a three-phase fluidized bed (Lertpocasombut, 1991).

Other experiments concerning the denitrification of drinking water were also conducted using a two phase fluidized bed reactor (Seropian et al., 1989 ; Lazarova et al., 1992). The results obtained show that it is possible to treat nitrate loadings of 3 kg N-NO_3/m^3.d over very long periods of operation.

Thus the O.S.B.G. materials in association with thin biofilms offer news perspectives for attached culture processes, notably attached culture processes with circulation of granular material.

8. Conclusion

The fundamental studies performed on the growth kinetics of aerobic and anaerobic biofilms have shown that the process can be described in six phases for the general case. After the latent phase which can be controlled through the surface properties of the support material, one notes a very fast dynamic phase, the end of which is characterized by the setting up of steady state functioning in the liquid phase. At this stage the biofilm is in the form of juxtaposed micro-colonies. Beyond this point the growth process slows down and an accumulation of biofilm at a constant rate is observed. In order to explain this phenomenon we suggest a new concept (M_a, M_d concept) which relies on the hypothesis that a biofilm is made up of two classes of bacteria : the active bacteria (M_a), situated at the biofilm/liquid interface and responsible for metabolizing the substrate, and the deactivated bacteria (M_d), situated inside the biofilm and responsible for its observed accumulation. After the linear phase the accumulation is slowed by loss of biofilm due to shear forces.

The study of the influence of various running conditions, notably the feed substrate concentration (S_0), shows a very strong dependence between the biological constants characteristic of the growth (μ_0 and K) and this parameter. This is notably seen from the increase in the substrate removal rate per unit volume (k_{0V}) with S_0, and the calculation of a constant and uniform active pseudothickness over the whole surface area (A_0) of the support leads to values of the order of 20 to 30 µm. In fact, since the biofilm/liquid exchange surface is much greater than A_0, the active pseudothickness is considerably less than 30 µm and the reaction tends towards a surface reaction. Thus, if the process is always reactive and if it takes place at the surface, it is necessary to control a) the initial stage of interaction between the micro-organisms and the support and b) the "active thickness" of the biofilm. For the case of processes employing cultures attached to a fine submerged granular medium, we have therefore developed a new material called O.S.B.G. (Optimized Support for Biological Growth) which has optimized surface and volume properties. The experiments underway at the moment, notably with fluidized beds, are encouraging from several points of view : purification, reactor stability, hydrodynamics, matter transfer and sludge production. The biomass concentrations reached at eliminated loadings of 10 to 15 kg TOC/m^3.d are only 0.5 kg/m^3 and sludge production is extremely low (0.1 to 2 kg D.W./kg eliminated TOD). These results show that a thin, uniform biofilm is the main objective to be attained.

9. Acknowledgements

The authors of this paper would particularly like to thank all the students of the "Unité de Recherche Traitement Biologique (URTB)" who have participated or are participating in the attached culture project. Our grateful thanks also go to the organizations and companies that have financed it : the Lyonnaise des Eaux Company, the National Agency for the Valorization of Research and the Ministry of Environment.

References

Atkinson, B. (1974) "Biochemical reactors", Pion Limited, London

Belkhadir, R. (1986) "Etude fondamentale des biomasses fixées : description et modélisation des films biologiques anaérobies", Ph. D. Thesis n°18, INSA Toulouse, France.

Belkhadir, R., Capdeville, B. and Roques, H. (1988) "Fundamental descriptive study and modelization of biological film growth", Wat. Res., 22, 1, 59-69.

Bird, R.B., Stewart, W.E. and Lightfoot, E.M. (1960) "Transport phenomena", John Wiley & Sons, Inc.

Capdeville, B. and Nguyen, K.M. (1990) "Kinetics and modelling of aerobic and anaerobic film growth", Wat. Sci. Tech., 22, 1/2, 149-170.

Capdeville, B., Aurelle, Y., Roustan, M. and Roques, H. (1988) "Matériau granulaire de traitement d'eau et procédé de fabrication", European Patent n°882003841.

Chang, J. (1988), "Etude cinétique et modélisation de la croissance d'une population mixte sur substrat complexe par couplage d'un réacteur discontinu à un spectromètre de masse", Ph. D. Thesis n°71, INSA Toulouse, France.

Characklis, W. G., Bakke, R. and Turakhia, M. H. (1988) "Theorical and experimental analysis of a Pseudomonas Aeruginosa biofilm", Wat. Sci. Tech., 20, 11/12, 45-51.

Characklis, W.G. and Marshall, K.C. (1990) "Biofilms", John Wiley & sons Inc., New York.

Characklis, W.G., Trulear, H.G., Bryers, J.D. and Zelver, N. (1982) "Dynamics of biofilm process : methods", Wat. Res., 16, 7, 1207-1216.

Christensen, F.R., Kristensen, G.H. and Jansen, J.L.C. (1988) "Biofilm structure : an important and neglected parameter in wastewater treatment", Wat. Sci. Tech., 21, 8/9, 805-814.

Cogny, M. (1987) "Mesures d'activités biologiques de cultures fixées", DEA, INSA Toulouse, France.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) "Colorimetric method for determination of sugars and related substances", Anal. Chem., 28, 3, 350-355.

Fletcher, M. (1980) "Adherence of marine microorganisms to smooth surfaces", Bacterial adherence, Beachy E.H. (ed.), Chapman et Hall, Londres.

Gaudy, A.F., Yang, P.Y., Bustamante, R. and Gaudy, E.T. (1973) "Exponential growth in systems limited by substrate concentration", Biotechnol. Bioeng., 15, 589-596.

Grady, C.P.L. and Lim, H.C. (1980) "Biological wastewater treatment : theory and applications", Marcel Dekker Inc., New York.

Hamoda, M.F. and Abd-El-Bary, M.F. (1987) "Operating characteristics of the aerated submerged fixed-film (ASFF) bioreactor", Wat. Res., 21, 8, 939-947.

Harremoes, P. (1977) "Half-order reactions in biofilms and filter kinetics", Vatten, 33,2,122-143.

Hatzifotiadou, O. (1989) "Contribution à l'étude de l'hydrodynamique et du transfert de matière gaz-liquide dans un réacteur à lit fluidisé triphasique", Ph. D. thesis n°83, INSA Toulouse, France.

Hatzifotiadou, O., Capdeville, B., Bataille, D., Roustan, M., Mauret, V., Bigot, V., Lertpocasombut, K. and Faup, G.M. (1988) "Hydrodynamique et transfert de matière dans un réacteur à lit fluidisé triphasique", Entropie, 143/144, 71-82.

Heim Da Costa, R. (1989) "Etude du contrôle du biofilm développé dans un lit fluidisé triphasique en épuration carbonée aérobie", Ph. D. thesis n°85, INSA Toulouse, France.

Hernandez, M., Vidard, V., Calvez, V., Aurelle, Y., and Capdeville, B., (1989) "Fundamental study of the interactions between microorganisms and plastic support", IAWPRC Conference on Technical advances in biofilm reactors, Nice, France, April, 565-566.

Huang, J., Chang, S. and Liu, Y. (1985) "Biofilm growth with sucrose as substrate", J. Environ. Eng., 111, 3, 353-363.

Kornegay, B.H. and Andrews, J.F. (1968) "Kinetics of fixed film biological reactors", J. Wat. Poll. Control. Fed., 40, 11, 460-468.

La Motta, E.J. (1976) "Kinetics of growth and substrate uptake in a biological film system", Applied and Environmental Microbiol., 31,2,286-293.

Lazarova, V., Capdeville, B. and Nikolov, L. (1992) "Biofilm performance of a fluidized bed biofilm reactor for drinking water denitrification", Wat. Sci. Tech., 26, 3/4, 555-566.

Lertpocasombut, K. (1991) "Epuration carbonée par film biologique mince dans un réacteur à lit fluidisé triphasique", Ph.D. Thesis n°159, INSA Toulouse, France.

Lowry, H.O., Rosebrough, N.S. and Farr Lewis, A. (1951) "Protein measurement with the folin phenol reagent", J. Biol. Chem., 193, 265-275.

Molin, G., Nilsson, I. and Stenson-Holst, L. (1982) "Biofilm build-up of *Pseudomonas putida* in a chemostat at different dilution rates", Europ. J. Appl.Microbiol. Biotechnol., 15, 218-222.

Monod, J. (1942), "Recherche sur la croissance des cultures bactériennes", Harmann et Cie (Ed.), Paris.

Nguyen, K.M. (1985) "Mise au point d'une méthode de dosage de l'activité déshydrongénasique", D.E.A., INSA Toulouse, France.

Nguyen, K.M. (1989) "Description et modélisation des films biologiques aérobies", Ph. D. Thesis n°96, INSA Toulouse, France.

Nouvion, N.(1985) "Etude de l'activité des microorganismes fixés dans des réacteurs à lit fluidisé en élimination de la pollution carbonée", Ph. D. Thesis, I.N.P. Lorraine, Nancy, France.

Ohgaki, S., Hwang, S.L. and Matzumoto, J. (1978) "Kinetics of attached microbial growth in a continuous stirred tank reactor", Wat. Res., 12, 243-249.

Revsbech, N.P. (1989) "Microsensors : spacial gradients in biofilms", in Structure and function of biofilms, W. G. Characklis and P.A. Wilderer, Eds, Dahlem Konferenzen, Berlin.

Rittmann, B.E. and Mac Carty, P.L. (1981) "Substrate flux into biofilms of any thickness", J. Env. Eng., ASCE, 107, 4, 831-850.

Rittmann, B.E. (1982) "The effect of shear stress on biofilm loss rate", Biotechnol. Bioeng., 24, 501-506.

Sanders W.M. (1966) "Oxygen utilization by slime organisms in continuous culture", Air and Wat. Pollut. Int. J., Pergamon Press, 10, 253-276.

Seropian, J.C., Vergne C., Moro A. and Capdeville, B. (1989) "Water denitrification : high efficiency biological fluidized bed", IAWPRC Conference on Technical advances in biofilm reactors, Nice, France, April, 545-546.

Trulear, M.G. and Characklis, W.G. (1982) "Dynamics of biofilm processes", J. Wat. Poll. Control Fed., 54, 9, 1288-1300.

Williamson, K. and Mc Carty, P.L. (1976) "A model of substrate utilization by bacterial films", J. Wat. Poll. Control Fed., 48,1,9-24.

MIXED POPULATION BIOFILMS

JAMES D. BRYERS
THE CENTER FOR BIOCHEMICAL ENGINEERING
DUKE UNIVERSITY
DURHAM, NORTH CAROLINA 27706 USA

1. Introduction

Multiple species biofilms comprise mixed bacterial populations within an extracellular polymeric matrix bound to a substratum. These attached mixed cultures are subject to interactions such as symbiosis or competition for either space or a common substrate; such interactions are directly or indirectly influenced by a myriad of variables associated with the surrounding environment. Spatial distributions of microbial populations are everchanging under the selection pressures exerted by processes such as: *(a)* exchange of bacterial species with the bulk liquid phase; *(b)* the relative efficiency of each species to metabolize their limiting substrate(s) into viable cell mass and non-viable extracellular polymers; *(c)* transport of limiting substrates and essential nutrients by molecular and convective transfer mechanisms; and *(d)* biofilm removal processes brought on by either physiological mechanisms (sloughing) or as a result of prevailing hydrodynamics (shear-related detachment). Evolution of spatial distributions of species within a biofilm can affect the biofilm's overall performance in specific situations (*e.g.,* natural purification of contaminated surface- or ground-waters, *in situ* bioremediation of xenobiotics, fate of recombinant DNA sequences and host microbes in the open environment, specific biofilm wastewater treatment systems, and specialty bioconversions). Consequently, it is critical to know how the ever-changing attached population distributions can affect overall system performance in order to better design, interpret, and operate biofilm systems.

2. Models of Biofilm Formation

Models which describe microbial growth either as an increase in total biomass or which consider only simple, single microbial conversion reactions are called *unstructured models.* Such lumped parameters approaches do not detail, for example, mixed culure population dynamics or changes in cellular composition in response to changes in environmental conditions. A structured model based on total cellular mass can not actually distinguish between pure and mixed cultures. Although relatively simple, these models have been applied throughout the industrial fermentation industry and the biological treatment sector verify experimental observations and provide initial process design parameters. Since such models are based simple biological conversion of substrate to cell mass, they are best suited for "balanced growth" steady-state systems.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 277–289.

Conversely, *structured models* do consider the additional detail of mixed culture population dynamics, cell molecular composition changes, and multiple reaction sequences. Consequently, a structured model is inherently better suited for simulating microbial physiology or mixed population dynamics under transient, unsteady conditions. While structured models provide more detail about a system, this necessitates a more phenomenological picture of the complex process interactions involved and quantitative information regarding process rate parameters. Historically, models of biofilm performance were initially unstructured, inductive "black-box" approaches that did not consider the *formation history* of a biofilm but rather simply the biofilm's efficiency in metabolizing a single limiting substrate. Evolution of our need to predict pure then mixed populations dynamics within an developing biofilm as led to a number of successive derivations that span the spectrum of unstructured to structured models as summarized by Wanner & Gujer (1986).

In the following, both modeling philosophies will be applied to biofilm formation beginning with a simplistic unstructured biofilm formation model followed by three different structured models; the first two structured models presume the biofilm is a well mixed continuum with no spatial distribution of species possible and the third, more complex model that accounts for spatial distributions of different species can arise in a developing biofilm.

2.1 AN UNSTRUCTURE MODEL OF BIOFILM FORMATION

2.1.1. *Basic Model Assumptions.* Unstructured versus structured modeling concepts are discussed in some detail in this volume's chapter on process analysis (Bryers and Characklis). Biofilm formation is assumed to occur on the exposed surface areas of a CSTR; thus, tacitly neglecting any spatial heterogeneity in the bulk liquid. Suspended biomass in the bulk liquid arises due to either cell growth and replication or due to shear-related erosion of biofilm material. Planktonic cells leave the liquid phase by either the effluent liquid leaving the reactor or by cell deposition onto the reactor surfaces. A single sterile growth limiting substrate, S, enters the reactor where it is consumed by either the suspended or biofilm-bound cells. Any reasonable measure of both biofilm and suspended cell concentrations is acceptable (cell number, biomass dry weight, biomass organic carbon) but note in the unstructured approach, no distinction between species or between cell carbon and EP carbon is made or required. However, one most know the stoichiometric relationship between changes in the suspended and attached biomass concentrations due to growth and the limiting substrate utilized.

2.1.2. *A General Unstructured Model.* Based on these assumptions and the scenario of biofilm formation detailed by Bryers and Characklis (this volume; process analysis), material balances for both the planktonic cell biomass and single growth limiting substrate can be written as Eqns. (1) and (2),

Suspended biomass balance:

$$VdX/dt = -FX + r_{gs}V + r_{ero}A - r_{dep}A \quad (1)$$

Limiting substrate balance:

$$VdS/dt = F(S_{in} - S) - [r_{gs}V/Y_{X/S}] - [r_{gb}A/Y_{B/S}] \quad (2)$$

where X = suspended cell biomass, S = limiting substrate concentration, V = reactor volume, $Y_{X/G}$ = yield coefficient for suspended cells, $Y_{B/S}$ = yield coefficient for biofilm mass, and A =

reactor area. Rate expressions for the remaining process rates (r_{gs}, r_{ero}, r_{dep}, r_{gb}) have been defined in the chapter on process analysis (Bryers & Characklis; this volume). with several depending directly on changing biofilm amount, limiting substrate and suspended biomass concentration. Should the reactor be operated at a residence time shorter than the generation time of the cells, then one can assume the term $r_{gs}V$ is negligible; however, the corresponding term in Eqn. (2) respresenting suspended cell substrate metabolism $[r_{gs}V/Y_{X/S}]$ can not be disregarded unless in the specific system it is determined small compared to the other terms.

Biofilm net accumulation can be described by Eqn. (3),

Biofilm net accumulation:

$$A\,[dm_f/dt = r_{dep} + r_{gb} - r_{ero}] \qquad (3)$$

Since rate expressions for both planktonic and biofilm growth are non-linear saturation kinetic functions of the instantaneous substrate concentration and first order functions of X and m_f, respectively, Eqns. (1) - (3) are coupled, non-linear ordinary differential equations that require either simultaneous numerical integration (for the transient situation) or simultaneous algebraic solution under conditions of steady-state (*i.e.*, dS/dt, dX/dt, and $dm_f/dt = 0$). Trulear (1983) determined, by independent experiments on a mixed culture biofilm, values for the various process rate kinetic constants which were then used to simulate changes in X, S, and biofilm thickness, y_f, in a rotating annular reactor operated at a residence time well in excess of the generation time for the culture (Figure 1). In this study, the biofilm thickness y_f was determined as the ratio of the biofilm area mass concentration to the biofilm density (m_f/ρ_f).

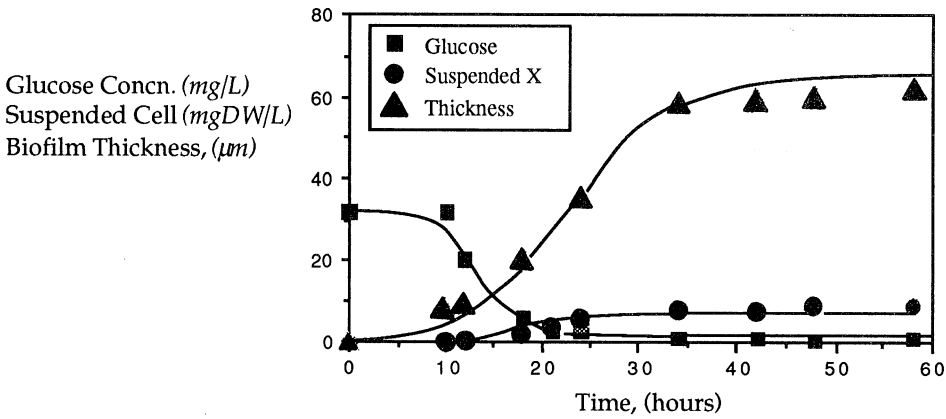


Figure 1. Progression of both liquid phase and surface parameters during mixed culture biofilm formation in a completely mixed rotating annular reactor operated at a dilution rate well in excess of the culture maximum growth rate. (Trulear, 1983). Lines represent predictions of a computer simulation of an unstructured model.

2.2 STRUCTURED MODELS OF BIOFILM FORMATION

A number of different structured models of biofilm formation exist that vary depending on how "structure" is applied to fulfill the needs of the research. Three will be detailed below; two models add structure to the biofilm composition yet treat it spatially as a well mixed continuum while a third model accounts for spatial distributions of both substrates and species within the biofilm.

2.2.1. *A Structured Model of Biofilm Composition.* Bakke *et al* (1984) presents a model of a pure culture biofilm system similar to that detailed in Section 2.1 above but in their model "structure" is added by considering the total biofilm amount per area, m_f, (measured as total organic carbon/area) as the sum of attached cell carbon/area, B, plus extracellular polymer carbon/area, EP_b, (*i.e.*, $m_f = B + EP_b$). A similar distinction is made for planktonic biomass (*i.e.*, $X = Xc + EP_s$). As in the unstructured model in Section 2.1.2 above, this model treats the biofilm as a well mixed reactor in that the components B and EP_b are assumed uniformly distributed throughout the depth of the biofilm; no spatial gradients in the various biofilm components are considered. By necessity, this structured model requires material balances for Xc, EP_s, and S in the liquid phase and B and EP_b in the biofilm phase. Similar equations to Eqns. (1) - (3) can be written that now require new process rate details regarding *(a)* the rate of EP production by both suspended and attached cells, *(b)* separate erosion rates for biofilm cells and EP_b, and *(c)* additional stoichiometric coefficients relating substrate conversion to both cell and EP production. The series of ordinary differential equations forming the structured model of a pure culture biofilm cell and EP components are summarized below:

Substrate Carbon Balance:

$$dS/dt\ [M_{sc}L^{-3}t^{-1}] = D(S_i - S) - \{\mu_b/Y_{b/s} + q_{pb}/Y_{ep/s}\}B - \{\mu_c/Y_{x/s} + q_{pc}/Y_{ep/s}\}X_c \quad (4)$$

Suspended Cell Balance:

$$dX_c/dt\ [M_{cc}L^{-3}t^{-1}] = -DX_c + r_{er/c}A - r_{dep/c}A + \mu_c X_c \quad (5)$$

Suspended Polymer Carbon Balance:

$$dEP_s/dt\ [M_{pc}L^{-3}t^{-1}] = -DEP_s + r_{er/p}A - r_{dep/p}A + q_p X_c \quad (6)$$

Biofilm Cellular Carbon:

$$dB/dt\ [M_{cc}L^{-2}t^{-1}] = \mu_b B + r_{dep/c} - r_{er/c} \quad (7)$$

Biofilm Extracellular Polymer Carbon:

$$dEP_b/dt\ [M_{pc}L^{-2}t^{-1}] = q_{pb}B - r_{er/p} + r_{dep/p} \quad (8)$$

where D = system dilution rate = flow rate F/reactor volume V; q_{pb} = specific polymer production rate for biofilm cells = $k\mu_b + k'$ where k is a growth related polymer production rate constant and k' is a non-growth related polymer production rate constant; a similar term, q_{pc}, exists for suspended cell polymer production rate; $Y_{x/s}$ and $Y_{b/s}$ are stoichoimetric coefficients for substrate carbon conversion to, respectively, suspended and biofilm cell carbon; $Y_{ep/s}$ = the stoichiometric coefficient for conversion of substrate to polymer; $r_{er/c}$ and $r_{er/p}$ are, respectively, the erosion rates for cells and polymer per area biofilm, $r_{dep/c}$ and $r_{dep/p}$

are, respectively, the net deposition rates of cells and polymer from the liquid onto the biofilm; and μ_b and μ_c are, respectively, the cell growth rates for biofilm and suspended cells. If the reactor in question is operated as a CSTR at a residence time less than the generation time of the cells, the last term in Eqns. (5) can be assumed negligible. Bakke *et al* (1984) used results from pure *Pseudomonas aeruginosa* biofilm experiments and steady-state versions of Eqns. (4) - (8), to determine the individual stoichiometric and kinetic parameters associated with the rate processes above. The reader should consult Bakke *et al* (1984 and 1990) for exact details of their experimental study and the different functions assumed for each rate expression above. Predictions of the above structured model compare well with experimental results of Trulear (1983) for a *Pseudomonas aeruginosa* biofilm cultivated in an annular rotating reactor (Figure 2).

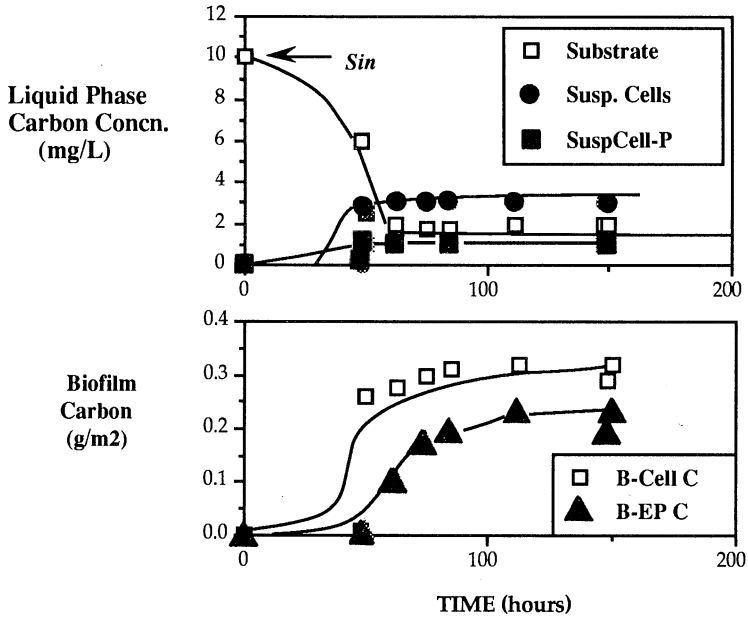


Figure 2. Progression of liquid and biofilm parameters for *Pseudomonas aeruginosa* biofilom formation in a rotating annular reactor. Lines represent predictions of a structured biofilm model.

2.2.2. *A Structured Model of a Mixed Culture Biofilm.* Bryers (1984) proposed a multiple species structured biofilm model that was a simple extension of the unstructured model in Section 2.1 that incorporated additional material balances per each species in both the biofilm and the fluid phase. Eqns. (4) - (8) can be repeated for each bacterial species identified, provided the individual kinetic and stoichiometric parameters for the new process rates are known. Either a separate growth limiting substrate can be assumed for each species necessitating additional material balances or, should the bacterial species compete for the same substrate, all that is required are additional substrate depletion terms per bacterial species in Eqn. (4). Models of this type are essentially the same as the two previous versions in that no consideration is given to any spatial segregation of cells or substrates within the biofilm. Siebel *et al* (1987) report on the use of just such a structured model of a two bacterial

species, *Pseudomonas aeruginosa* and *Klebseilla pneumoniae* , each competing for the same electron donor (glucose) and acceptor (oxygen) within a rotating annular biofilm reactor. Values for the individual rate constants for the processes of deposition, cell growth, and biofilm erosion were determined experimentally from pure culture biofilm formation studies similar to those depicted in Figure 2. Using the parameters from the pure culture studies, a binary population model was employed to simulate the formation of the mixed culture biofilm; a comparison of predictions and observations are provided in Siebel *et al.* (1987).

3. Modeling Spatial Distributions in Multiple Species Biofilms

A number of existing complex models can predict the development of spatial distributions of different bacterial species within a developing biofilm competing for multiple growth limiting substrates (Wanner & Gujer, 1986; Kissel *et al.* , 1984). The version of Wanner & Gujer (1984) will be used here; for details regarding the derivation of their original model and subsequent modifications the reader is directed elsewhere (Gujer and Wanner, 1990; Gujer, 1987).

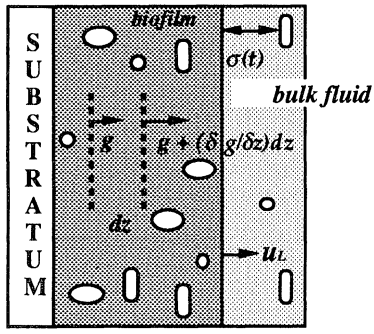


Figure 3. Flux of species biomass in an expanding biofilm.

A number of fundamental assumptions are necessary in order to model a mixed culture biofilm developing in a direction perpendicular to the plane of the substratum (Figure 3). The model is based on assumptions that *(a)* properties of the biofilm - *i.e.*, bacterial species and substrate concentrations - change only in the direction perpendicular to the substratum and *(b)* that the biofilm is a continuum. Biomass of each species "moves" due to convective motion of that species' mass at a velocity dependent not only on the cells growth rate at a point in the biofilm but also due to displacement by neighboring cells. Dissolved components in the liquid phase are assumed to diffuse within the biofilm by molecular diffusion assuming a constant diffusion coefficient; (*i.e.*, Fick's law prevails within the biofilm). *(c)* The biofilm overall volumetric density is assumed to remain constant. *(d)* The model is capable of accounting for both extracellular polymers and dead cell mass if desired. *(e)* Growth rate dependencies of each species on its limiting substrate(s) can be any type of functional dependency (*e.g.*, Monod, dual-Monod, Blackman kinetics) and will vary locally with time as the biofilm develops. Finally, *(f)* exchange of cells and dissolved components in the fluid phase with the biofilm can be linked with a material balance over the reactor volume by assuming the biofilm develops within a well-mixed CSTR.

A mass balance on microbial species i set up over a differential element of the biofilm can be written as,

$$\partial f_i/\partial t = \mu^o{}_i f_i - (1/\rho_i)\, \partial g_i/\partial z \qquad (9)$$

where ρ_i = the constant density, f_i = the volume fraction, $\mu^o{}_i$ = the net specific growth rate, and g_i = the mass of bacterial species i that is displaced per unit time and area relative to the

substratum. The mass flux g_i of species i can also be expressed as the product of the velocity u at which microbial mass is displaced and its mass concentration,

$$g_i\,(t,z) = u(t,z)\rho_i f_i(t,z) \tag{10}$$

The velocity$u$ is unknown but can be determined by relating the biofilm volume expansion to biomass production. Summarizing the results of Wanner and Gujer (1986), the mean observed growth rate of biomass, μ_o, is

$$\mu_o(t,z) = \partial u(t,z)/\partial z \tag{11}$$

Integrating to solve for the velocity yields,

$$u(t,z) = \int_0^z \overline{\mu_0}(t,z')\ dz' \tag{12}$$

If the biofilm grows or shrinks, the thickness L changes and the biofilm-fluid interface moves at a velocity u_L ,

$$u_L = dL(t)/dt \tag{13}$$

Defining $\sigma(t)$ as the rate at which biomass is exchanged between the biofilm and the fluid interface, then the velocity of the biofilm-fluid interface can also be expressed as,

$$u_L(t) = \int_0^L \overline{\mu_o}(t,z')dz' + \sigma(t) \tag{14}$$

In a similar fashion, a mass balance for any substrate *i*, can be written as,

$$\partial S_i(t,z)/\partial t = r_i(t,z) - \partial j_i(t,z)/\partial z \tag{15}$$

where S_i = the local, instantaneous concentration, j_i = the mass flux, and r_i = the observed biological conversion rate of substrate i. The mass flux of substrate can be related to the concentration gradient by Fick's first law,

$$j_i(t,z) = - \mathcal{D}_i\, \partial S_i(t,z)/\partial z \tag{16}$$

The reaction rate expressions depend upon the microbial conversion and bacterial species in question. Reaction rate expressions take the form of dual substrate limited saturation kinetics minus an oxygen dependent endogenous decay rate,

$$r_i\,(t,z) = \mu^*_i\rho_i f_i[S_{O2}/(K_{O2} + S_{O2})]\,[S_{EDi}/(K_{EDi} + S_{EDi})] - k_e\rho_i f_i[S_{O2}/(K_{O2} + S_{O2})] \tag{17}$$

where μ^*_i = the maximum growth rate constant for species i ; S_{O2} = the local instantaneous concentration of oxygen (which is the common terminal electron acceptor for the ecology considered in this study); K_{O2} = the saturation rate constant; k_e = the endogenous decay rate constant for species i; and S_{EDi} and K_{EDi} = the local, instantaneous concentration and the saturation rate constant for the specific electron donor used by species i.

In one study, Gujer and Wanner (1990) considered populations of heterotrophic carbon-oxidizing bacteria competing with autotrophic nitrogen-oxidizing species. In the case of the heterotrophs, the electron donor is organic carbon, for *Nitrosomonas spp.* the electron donor is ammonia nitrogen (represented here as NH_4^+-N), and Nitrite nitrogen (NO_2^--N) for *Nitrobacter spp.* Note: the oxygen dependent endogenous decay rate expression is identical to that in the growth rate term which, should the oxygen at some depth in the biofilm go to zero, renders the overall reaction zero at that (t,z). While this computationally prevents negative reaction rates, it also tacitly ignores the potential for denitrification by facultative heterotrophs; the biofilm model of Kissel *et al.* (1984) does account for possible denitrification. Bryers and Banks (1990) used the Gujer-Wanner model to simulate biofilm development of two heterotrophs, a *Hyphomicrobium spp.* growing exclusing on methanol and a *Pseudomonas putida* metabolizing exclusively glucose, both competing for oxygen.

Maximum overall uptake of any substrate varies with time as the biofilm increases (or decreases) and as the bacterial populations change; this maximum observed uptake rate per unit area of biofilm can be calculated as the flux of substrate evaluated at $z = L$. Bacterial species and substrate profiles in the developing biofilm can be numerically determined using the *methods of lines* as detailed in Wanner and Gujer (1986).

3.1 COMBINED NITRIFICATION:CARBON OXIDATION BIOFILMS

Wanner and Gujer (1986) present an illustration of the utility of the multiple species biofilm above for the competition between autotrophic nitrifying bacteria oxidizing ammonia and nitrite nitrogen and heterotrophic carbon-oxidizing bacteria. An inert fraction of the biofilm was also modeled to represent polymer production and inert dead cell material. Dissolved components considered were organic carbon (as COD), NH_4^+-N, and O_2. In the following example, reactor inlet (and initial) conditions in the fluid phase were 3 $(gCOD)m^{-3}$, 13 $(gNH_4^+\text{-}N)m^{-3}$, and 8 $(gO_2)m^{-3}$. Biofilm thickness was initially set at 5 μm and the various biofilm components set at 0.0 inert fraction, 0.65 heterotrophs, and 0.35 autotrophs; expressed as fractions of the total biofilm density here assumed to be 35 g cm^{-3}. Figure 4 illustrates predictions of the Wanner & Gujer model for the progression of biofilm thickness and species distrubution with depth for the first ten days of the development.

Bryers and Banks (1990) employed the above model under slightly different conditions to simulate experimental observations a nitrifyier:heterotroph biofilm. Parameters employed for the computer simulations are provided in Table 1. Predicted fractions of biofilm species were compared to the concentrations determined for both HET and NTF using dual radiolabelling of the biofilm bacteria. Heterotrophs were radiolabelled with ^{14}C-acetate (0.5 μCi/mL) while autotrophic nitrifiers were labelled with $NaH^{14}CO_3$ (58μCI/mmol) followed by microtome

sectioning of the biofilm at 20μm depth intervals parallel to the plane of the substratum. For details regarding these experiments the reader is directed to Bryers and Banks (1990).

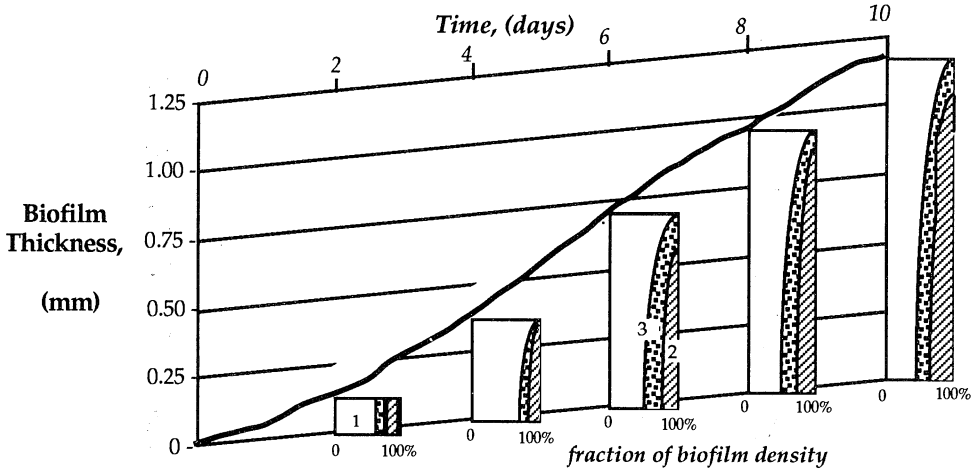


Figure 4. Model predictions of hetrotrophic:nitrifier biofilm species profiles. Fraction of biofilm density of each species is based on a total biofilm density of 35 g cm^{-3}. Feed conditions are stated in the text above. Fractions considered a part of the biofilm mass are: (1) heterotrophs, (2) nitrifiers, and (3) exopolmyers. Wanner and Gujer (1986).

Experimental disintegrations per minute were converted to cell numbers then to cell carbon by calibration curves for each species. Predicted species concentrations in the Wanner-Gujer model were converted from fractions of the biofilm density to mass-carbon areal concentrations using the experimentally determined biofilm density. A comparison of predicted and observed biofilm species distributions are illustrated in Figure 5A&B for two different experimental conditions: a situation to promote nitrifyier dominance (NH_4^+-N = 5.0; O_2 = 8.5; and Acetate = 1.0 ppm) and one to promote heterotroph dominance (NH_4^+-N = 5.0; O_2 = 8.5; and Acetate = 3.0 ppm).

Table 1. Parameters Employed in Computer Simulations of Multiple Species Biofilms

		Ecosystem I		Ecosystem II	
PARAMETERS		Heterotrophs	Nitrifiers	Ps. putida	Hyphomicro.
μ*	(hr^{-1})	0.2	0.039	0.54	0.24
K_S	(mgS/L)	5 (acetate)	1 (ammonia)	10.0 (C6)	0.01 (C1)
K_{O2}	(mgO_2/L)	0.1	0.1	1.0	0.10
$Y_{x/s}$	(mgX/mgS)	0.45	0.10	0.42	0.30
k_e	(h^{-1})	0.008	0.004	0.005	0.002
D_{eff}	(cm^2/sec)	acetate:	9.6E-06	methanol:	15.0E-06
		ammonia:	17.2E-06	glucose:	5.0E-06
		oxygen:	20.2E-06	oxygen:	20.2E-06

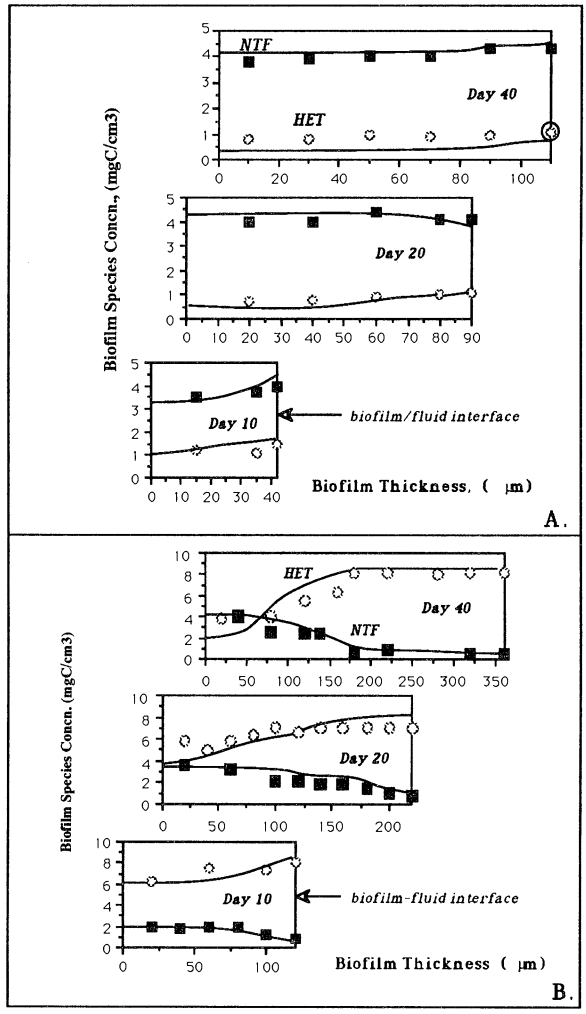


Figure 5. Comparison of computer simulation using Gujer-Wanner model to mixed heterotrophic-autotrophic culture biofilm experiments. (**A.**) NH_4^+-N = 5.0; O_2 = 8.5; and Acetate = 1.0 ppm. (**B.**) NH_4^+-N = 5.0; O_2 = 8.5; and Acetate = 3.0 ppm.

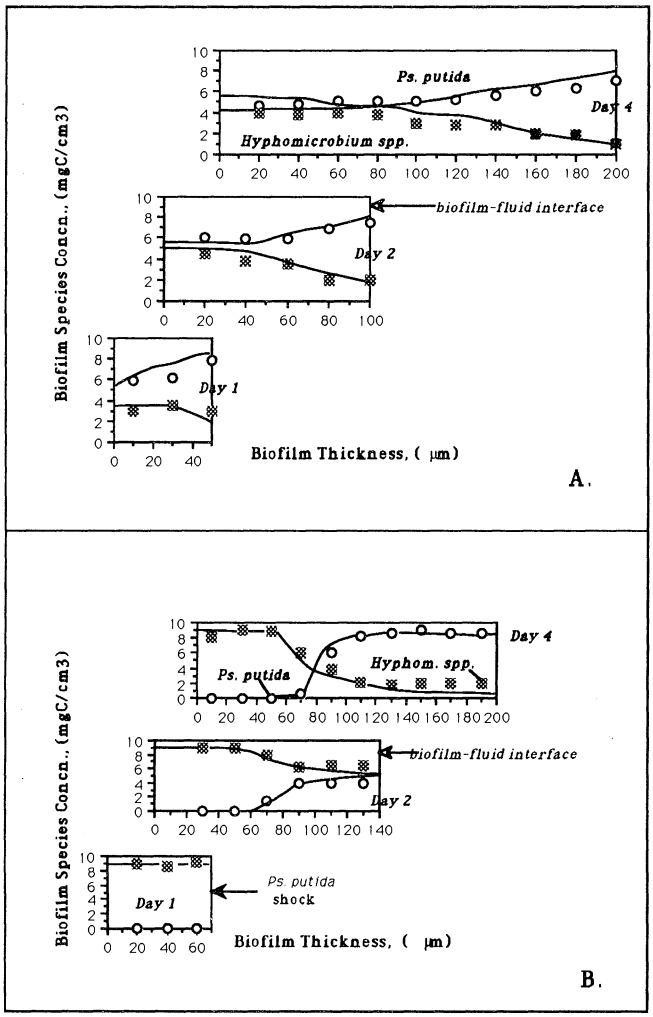


Figure 6. Comparison of computer simulation using Gujer-Wanner model to a binary heterotrophic mixed culture biofilm experiment. (**A.**) Both *Pseudomonas putida* and *Hyphomicrobium spp.* seeded on surface at same time; nutrient conditions detailed in text. (**B.**) *Hyphomicrobium spp.* pure culture biofilm grown for one day, exposed to *Pseudomonas putida* suspension for 2 hours, then biofilm fed as in A. An artificial bi-layer biofilm is created.

Bryers and Banks (1990) also simulated a second system, also summarized in Table 1, of two chemoheterotrophs metabolizing separate electron donors but both competing for oxygen. *Pseudomonas putida* ATCC 11172 metabolized exclusively glucose while *Hyphomicrobium spp. ZV620* grew only on methanol. Subsamples of the same biofilm sample could thus be exposed to either ^{14}C-glucose or ^{14}C-methanol to label the separate species. Samples were fixed, embedded, and sectioned in 20 μm thick increments parallel to the plane of the substratum as describe in Bryers and Banks (1990). A comparison of predicted and observed biofilm speciesdistributions are illustrated in Figure 6A&B for two different experimental conditions: *(1)* an artificially constructed bi-layer biofilm where a pure culture of *Hyphomicrobium spp.* is overgrown with *Pseudomonas putida* and then both electron donors suppled and *(2)* where both *Pseudomonas putida* and *Hyphomicrobium spp.* are inoculated at time equal zero and grown on equal concentrations of glucose and methanol; the biofilms were oxygen limited.

4. Summary

Biofilm systems are very complex. Often times out of experimental expediency, research simplifies these complexities by dealing with *in vitro,* well-controlled laboratory systems that use pure bacterial cultures. Such environmentally controlled systems have in the past provided much information on the dynamics of biofilm formation and activity. However, only in rare situations are the results of such pure culture work additive; mixed population biofilms pose additional nuances on the structure and function of biofilms that can not be garnered from pure culture systems. This lecture will exemplify a number mixed population bacterial biofilm systems. Effects of mixed population biofilms on our understanding of biofilm structure, mass transport properties of the biofilm matrix, and morphological characteristics of the biofilm will be outlined. Mathematical modeling of population distributions within developing biofilms will be presented and experimentally verified. Models presented could be extended to other mixed cell biofilms including multiple species or strains, mixed procaryotic-eucaryotic biofilms, and mixed cell line (bacteria, platelets, endothelial cell) biofilms.

5. References

Bakke, R., Trulear, M.G., Robinson, J. A., and Characklis, W.G. (1984) Activity of *Pseudomonas aeruginosa* in Biofilms: steady-state, *Biotechnol. Bioengrg.*, **26**, 1418-1424.

Bryers, J. D. (1984) Biofilm formation and chemostat dynamics: pure and mixed culture considerations, *Biotechnol. Bioengrg.* **26**, 948-958.

Bryers, J. D. and Banks, M. K. (1990) Assessment of biofilm ecodynamics, in J.A.M. de Bont, J. Visser, B. Mattiasson, J. Tramper (Eds.) *Physiology of Immobilized Cells*, Elsevier Publ., Amsterdam, 49-62.

Gujer, W. (1987) Significance of segregation of biomass in biofilms, *Water Sci. Technol.*, **19**, 495-503.

Kissel, J. C., McCarty, P. L. and Street, R.L. (1984) Numerical simulation of a mixed culture biofilm, *J. Envir.Engrg., ASCE*, **110**, 393.

Siebel, M. A. (1987) *Binary Population Biofilms*, Ph.D. Dissertation, Montana State University, Bozeman, MT.

Trulear, M. G. (1983) *Cellular Reproduction and Extracellular Polymer Formation in the Development of Biofilms*, Ph.D. Dissertation, Montana State University, Bozeman, MT.

Wanner, O. and Gujer, W. (1986) A multispecies biofilm model, *Biotechnol. Bioengrg.*, **28**, 314-328.

ACKNOWLEDGEMENTS

JDB would like to thank both the National Science Foundation (BCS-9020502) and the NATO Advanced Studies Institute for their financial and intellectual support of this effort.

DYNAMIC MODELING THE GROWTH OF IMMOBILIZED NITRIFYING BACTERIA : BIOFILM DEVELOPMENT.

C.D. DE GOOIJER, R.H. WIJFFELS, and J. TRAMPER
Wageningen Agricultural University
Food and Bioprocess Engineering Group
P.O. Box 8129, 6700 EV Wageningen, The Netherlands.

1 Introduction

The use of immobilized growing cells has become of increasing interest in the past few years. Traditionally, biofilms are used in wastewater treatment (Canovas-Diaz and Howell 1988, Saez and Rittmann 1988, Suidan et al. 1987, Siegrist and Gujer 1987). In this field the use of entrapped nitrifying bacteria is gaining importance, as this will lead to a more stable and well-defined system (Wijffels and Tramper 1989). The overall volumetric productivity of bioreactors can be largely enhanced by the application of immobilized cells. The dilution rate can be very high since cells will not be washed out of the reactor. The growth rate of nitrifying bacteria is always low and in particular the application of immobilized cells is advantageous.

The combination of mass transport by diffusion and simultaneous substrate consumption is described in numerous papers. Steady-state models for growing cells (Suidan et al. 1987, Monbouquette and Ollis 1986, Andrews 1988) can be quite satisfactory for design purposes, but may fail to describe the start-up phase and the response to changing conditions in the reactor. Only a few are capable of describing transient states, since the majority of the papers assume steady-state with a homogeneous biomass distribution, exclude growth phenomena, and/or omit external diffusion limitation (Nakasaki et al. 1989, Sayles and Olis 1989, Monbouquette et al. 1990).

There are three rationales for a dynamic model : a dynamic model will contribute to the understanding of the immobilized-cell process, it can be used to predict the dynamic response of the system to a change in environmental conditions, and it can be used as a tool in bioreactor design.

In this paper we describe the development of a model that is capable to describe simultaneous internal and external mass transport, substrate consumption, and biomass growth. The model describes the occurrence of an inhomogeneous biomass distribution with time (de Gooijer et al. 1991). Since it is based on non-specific bacterial kinetics and substrate transfer by diffusion, it can be used in a general way. The model predicts substrate and biomass profiles across immobilization supports, and, from that, the overall substrate consumption rate by the immobilized biomass. This model was evaluated with *Nitrobacter agilis* cells immobilized in spherical particles of κ-carrageenan (Wijffels et al. 1991). With oxygen as the limiting substrate, the model was shown to be able to describe very satisfactorily the oxygen consumption rate for both the transient- and the steady-state at three different bulk oxygen concentrations.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 291–296.

2 Theory

In many aerobic systems oxygen is the limiting substrate, due to the rather low solubility in water. In a previous paper (Wijffels et al. 1991) we have shown that this is also the case for nitrifying bacteria, and that both internal and external diffusion limitation are likely to be rate-limiting.

In the case of growing immobilized cells, substrate depletion near the centre of the biocatalyst bead will occur sooner or later, whereas a high substrate concentration will be available near the surface. Therefore a growth model is needed that is capable of describing both situations, i.e. allowing a negative net growth at low substrate concentrations, and a maximum growth rate at high substrate concentrations. For that we use the growth model suggested by Beeftink et al.(1990) :

$$r_s = \frac{\mu}{Y_{xs}} X + mX\left(\frac{S}{K_s + S}\right) \tag{1}$$

$$r_x = \mu X - mY_{xs}X\left(1 - \frac{S}{K_s + S}\right) \tag{2}$$

This is a combination of the models of Pirt (1965) and Herbert (1958). The specific growth rate constant μ is described by the Monod equation.

Many researchers proved that the effective diffusion coefficient is affected by the concentration of biomass in the support material (Canovas-Diaz and Howell 1988, Andrews 1988, Wijffels et al. 1991). In order to account for the dependency of the effective diffusion coefficient on the biomass concentration, we implemented a relationship that generates an effective diffusion coefficient that varies linearly with the biomass. The effective diffusion coefficient will become equal to the diffusion coefficient in the gel if no biomass is present, and zero when all available space in the gel bead is occupied by biomass. This is described by :

$$D_e = D_{e,g}(1 - X/X_{pmax}) \tag{3}$$

The well-known differential equation for simultaneous consumption and diffusion of substrate is given by:

$$D_e\left(\frac{1}{r^2}\frac{d}{dr}\left(r^2\frac{dS}{dr}\right)\right) = r_s + \frac{dS}{dt} \tag{4}$$

with the boundary conditions:

$S = S_i$ at r = r_b, and $\frac{dS}{dr} = 0$ at r = 0, or r = r_f

In order to account for external mass-transfer resistance, we used a combination of Fick's law and the film theory :

$$S_i' = S_b - \frac{D_e}{k_{l,s}}\frac{dS}{dr}\Big|_{r=r_b} \tag{5}$$

A straightforward application of the differential equations would lead to infinitively high biomass concentrations near the surface of the bead. As this is impossible, a restriction was added to this equation :

$$X \leq X_{max} \tag{6}$$

This set of equations was solved numerically as described before (de Gooijer 1991).

In a first extension of this model, the observation was implemented that biomass will grow in colonies inside the gel bead as described by Salmon (1989). In that case the biomass in the beads consists of a collection of colonies with different radii. The colony radius is determined by the radial position of the colony in the bead, and colonies at a certain radial position have identical diameters. Then, the volume of bacterial colonies in $0 \leq r \leq r'$ (see figure 1) is given by

$$v(r') = \frac{3n}{r_b^3} \int_0^{r_b} r^2 \omega(r, r') dr \tag{7}$$

where $\omega(r,r')$ is the volume of a colony at radial position r ($0 \leq r \leq r'$), and n the number of colonies in one bead. In calculating $\omega(r,r')$, three cases must be considered as illustrated in figure 1.

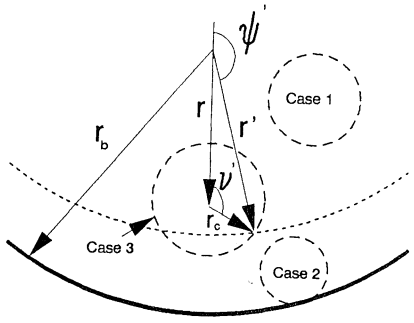


Figure 1. Symbols used for calculation of colony growth. (Adapted from Salmon 1989).

Case 1. $r \leq r' - r_c(r)$. In this case the entire colony will be situated in the area between $0 \leq r \leq r'$, and thus $\omega(r,r') = 4/3\ \pi\ (r_c)^3$.

Case 2. $r \geq r' + r_c(r)$. Here, the colony is situated entirely outside the area of interest, so that $\omega(r,r') = 0$.

Case 3. $r' - r_c(r) < r < r' + r_c(r)$. Part of the colony is situated in the area of interest. For this situation Salmon (1989) derived that

$$\omega(r,r') = \frac{\pi r_c^3}{3}(2-(3-\cos^2\nu')\cos\nu') + \frac{\pi r^3}{3}(2+(3-\cos^2\Psi')\cos\Psi') \tag{8}$$

in which $\cos\nu' = (r_c^2 + r^2 - r'^2)/(2r_c r)$ and $\cos\Psi' = (r_c^2 - r^2 - r'^2)/(2r'r)$. To be able to calculate the biomass concentration at a radial position in the bead, we use

$$X = \rho_x \frac{v(r'_n) - v(r'_{n-1})}{r_n^3 - r_{n-1}^3} \tag{9}$$

in which ρ_x is the biomass density, and n denotes the area number.

After some time colonies reach the surface of the beads. At this point two different mechanisms were implemented. The first is based on the assumption that the colonies

remain intact, and that further biomass growth is discharged into the medium. With the second mechanism it is assumed that at the moment the colony reaches the bead surface, its entire content is washed out into the medium.

3 Results and Discussion

A typical result of both the normal and the extended model is shown in Figure 2. In the transient growth of immobilized cells, 4 phases can be recognized. In phase 1, there is hardly any diffusion limitation, and homogeneous growth occurs. Phase 2 shows inhomogeneous growth, as in the centre of the sphere substrate depletion aggravates. In phase 3, the substrate hardly reaches the core of the sphere anymore, so biomass decays, where at the outer shell the biomass concentration reaches its maximum. In the extended model, the biomass colonies reach the outer shell of the bead and biomass eruption takes place. Consequently, the biomass concentration at the surface of the bead drops. The difference between the two methods of biomass discharge is negelectable.

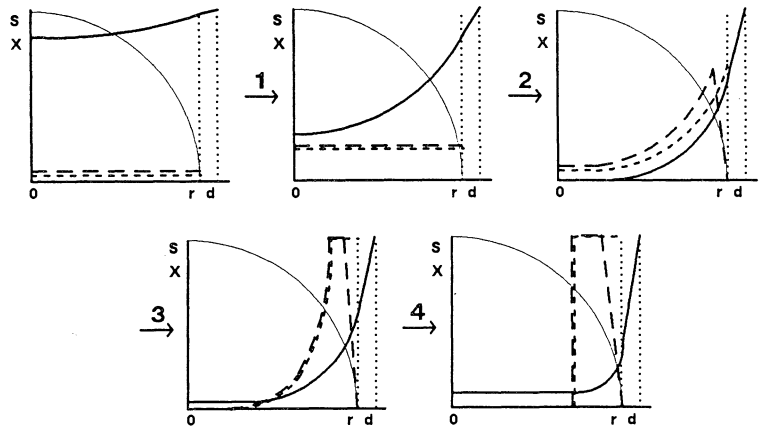


Figure 2. Typical model predictions for growth of immobilized cells. Substrate concentrations (—) and Biomass concentrations (--- = base model, — — = extended model) versus radial position r at various times. The external stagnant layer is represented by d.

In phase 4 this process of growth and decay continues and a distinct biomass film developes, whereas the substrate concentration in the centre of the sphere increases. At the end time of the simulation a distinct biomass film near the surface of the bead has developed, whereas the substrate concentration inside the bead corresponds to the maintenance level of zero net growth.

The model was verified with *Nitrobacter agilis* cells immobilized in κ-carrageenan, as presented by Wijffels et al. (1991).

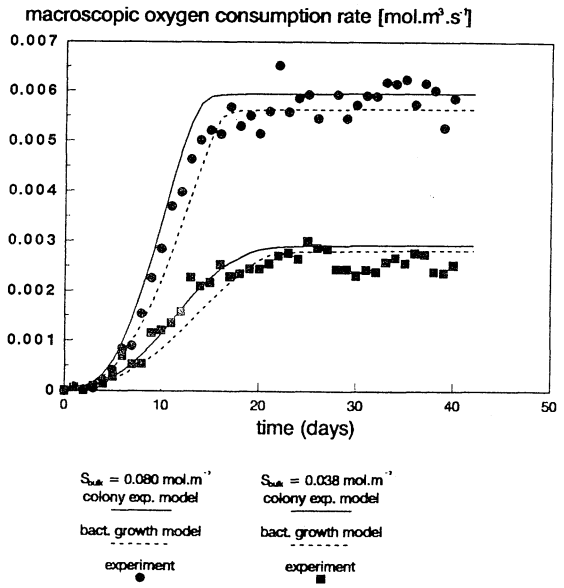
Immobilized cells were kept in an air-lift loop reactor at sufficiently high nitrite concentrations and constant oxygen concentrations, so that oxygen was the limiting substrate. At two different oxygen concentrations the macroscopic oxygen consumption rates were determined in time by measuring the nitrite consumption rate, and compared to model predictions. Parameters used in the model are given in Table 1, and results are shown in

Figure 3. The curves predicted by both models match the experimental results very well. As can be seen from figure 4, the simulated biomass profile improved with the use of the extended model.

Parameter	Value	Unity	Parameter	Value	Unity
$k_{l,s}$	$3.7*10^{-5}$	m/s	Y_{xs}	$1.16*10^{-3}$	kg/mol
$D_{e,g}$	$1.58*10^{-9}$	m^2/s	μmax	$1.0*10^{-5}$	1/s
r_b	$1.02*10^{-3}$	m	K_s	$1.7*10^{-2}$	mol/m^3
X_0	$4.5*10^{-3}$	kg/m^3	m	$1.1*10^{-3}$	mol/kg.s
X_{pmax}	950	kg/m^3	X_{max}	11	kg/m^3
t_{end}	42	day	S_b	3.8/8.0	10^{-2} mol/m^3
Δt	1	day			

Table 1. Parameters used in the model evaluation (From Wijffels et al. 1991). Here, Δt is the time step for the growth process (equation 2), and X_0 is the initial biomass concentration.

Figure 3. Experimental evaluation of the model. Macroscopic consumption rates at three different oxygen concentrations versus time. Lines are model predictions (—— = base model, --- = extended model), markers are experimental data. Experiments were performed at oxygen bulk concentrations of $3.8*10^{-2}$ $mol.m^{-3}$ (squares), and $8.0*10^{-2}$ $mol.m^{-3}$ (circles).



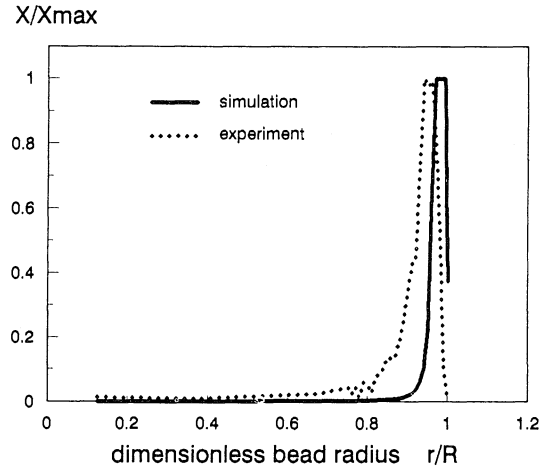


Figure 4.
The dimensionless biomass concentration versus radial position. The biomass profile as determined by Wijffels et al. (1991) (—) and the biomass concentration as predicted by the extended model (---).

4 Conclusions

The modeling of immobilized *Nitrobacter agilis* cells was successfully carried out. Measured macroscopic oxygen consumption rates matched well with model predictions at three different oxygen concentrations. Figure 2 shows that external diffusion limitation is of high importance in the steady-state situation. Currently we are extending the model by incorporating diffusion limitation over the micro-colonies. Furthermore, the model is integrated with a flow model to an overall reactor model.

5 References

Andrews G (1988) Chem Eng J 37:b31-b37
Beeftink HH, van der Heijden RTJM, Heijnen JJ (1990) FEMS Microbiol Ecol 73:203-209
Canovas-Diaz M, Howell JA (1988) Biotechnol Bioeng 32:348-355
de Gooijer CD, Wijffels RH, Tramper J (1991). Biotechnol Bioeng 38:224-231
Herbert D (1958) in : Recent progress in microbiology, Ed. Tunevall G, Almqvist & Wiksell, Stockholm 381-396
Monbouquette HG, Ollis DF (1986) Ann NY Acad Sc 469:230-244
Monbouquette HG, Sayles GD, Ollis DF (1990) Biotechnol Bioeng 35:609-629
Nakasaki K, Murai T, Akiyama T (1989) Biotechnol Bioeng 33:1317-1323
Pirt SJ (1965) Proc Roy Soc London 163 B, 224-231
Saez PB, Rittmann BE (1988) Biotechnol Bioeng 32:379-385
Salmon P.M. (1989) Mass transport phenomena in reactors containing entrapped enzymes or bacterial cells. PhD Thesis, Stanford University, USA.
Sayles GD, Ollis DF (1989) Biotechnol Bioeng 34:160-170
Siegrist H, Gujer W (1987) Wat Res **21** 12:1481-1487
Suidan MT, Rittman BE, Traegner UK (1987) Wat Res **21** 4:491-498
Wijffels RH, Tramper J (1989) Appl Microb Biotechnol 32:108-112.
Wijffels RH, de Gooijer CD, Kortekaas S, Tramper J (1991). Biotechnol Bioeng 38:232-240

Chapter 5

BIOFILMS IN MEDICINE

BACTERIAL COLONIZATION OF IMPLANTS: BIOFILMS AND URINARY CATHETERS

MADILYN FLETCHER
Center of Marine Biotechnology
Maryland Biotechnology Institute
University of Maryland System
600 E. Lombard St.
Baltimore, Maryland 21202
USA

1. Introduction

The development and use of biomaterials is one of the most important advances of modern medicine. The replacement of diseased or damaged body parts by artificial implants, e.g., joint or vascular prostheses, has restored the health of enormous numbers of individuals. Many more temporary devices, e.g., urinary catheters, intravascular catheters, endotrachael tubes, are inserted into patients for various lengths of time. A numbers of materials are used for such devices, including titanium alloy, cobalt-chromium-molybdenum alloy, and organic polymers (e.g., polytetrafluoroethylenes, polyethylene) (Dickinson and Bisno, 1989), and all of these can serve as substrata for bacterial biofilms. If bacteria reach these surfaces *in vivo*, either through the blood stream, via a conduit to the outside of the body (e.g., as with a catheter), or by original contamination of the device, then attachment to the surface and biofilm development can occur. Indeed, the major complication of use of implants and prosthetic devices is bacterial infection (Dickinson and Bisno, 1989).

A variety of bacteria are involved in the colonization of implants. These include both Gram-negative organisms, e.g., *Pseudomonas aeruginosa* and *Escherichia coli*, and Gram-positive bacteria, e.g., *Staphylococcus aureus* and *S. epidermidis*. This review will focus on the colonization of urinary catheters by primarily Gram-negative bacteria, but many of the general observations made with these systems apply to other types of implant colonization.

2. Bacterial Colonization of Urinary Catheters

Long-term catheterization results in bacterial infection of the urinary tract (Warren et al., 1987), because the catheter serves not only as a conduit for infection from outside

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 299–308.

sources, but also as a colonization site for bacteria. Here they can resist wash-out by urine flow and antibiotic treatment (Dickinson and Bisno, 1989; Gristina, 1989). In addition to providing a colonization site, hence focus for infection, the catheter may also damage epithelium mucosa, allowing bacterial invasion into deeper mucosal layers.

Certain species have been consistently isolated from long-term catheter patients with bacterial infection of the urinary tract (bacteriuria). Particularly common are Gram-negative rods, such as *Providencia stuartii*, *Proteus mirabilis*, *Escherichia, coli*, *Pseudomonas aeruginosa*, *Morganella morganii*, and *Klebsiella pneumoniae* (Warren 1986; Jewes et al., 1988; Stickler et al., 1988; Tenney and Warren, 1988). However, the relative numbers of these bacteria in the urine are probably not a true reflection of their numbers on the catheter surface. The abundance of bacteria in the liquid is influenced not only by the size of the bacterial reservoir on the surface, but also by the degree to which the different bacterial strains detach from the biofilm and are released into the urine.

Since the colonization of catheters is invariably polymicrobial, i.e. involves more than one species, interactions among those species determine biofilm ecology in several ways. First, interactions can influence the initial attachment process (McEldowney and Fletcher, 1987; Kolenbrander, 1988). For example, interactions among different isolates from a food processing plant affected the attachment of the individual species to glass, nylon, and steel surfaces (McEldowney and Fletcher, 1987). One species facilitated the adhesion of others, whereas other species interfered with attachment. The type of interaction depended on both the combination of species and chemical composition of the attachment surface. Similarly, *Lactobacillus* cells or cell wall fragments interfered with adhesion of uropathogens to human uroepithelial cells (Chan et al., 1985). Second, as the cells grow and the biofilm develops, interactions among species can stabilize the structure mechanically. In biofilms in the mouth, specific adhesive interactions have been identified, such that different genera coaggregate due to the interaction of cell surface molecules (Kolenbrander, 1988). Finally, competition for substrate and production of metabolites will also influence the colonization success of bacteria within a biofilm. For example, the production of urease by an organism in the urinary tract would lead to an increase in pH, which would influence growth of other colonizing bacteria (McLean et al., 1988). Thus, to understand polymicrobial colonization of the catheter surface, it is clearly essential to understand the interaction of organisms in mixed infections.

3. Factors Affecting Adherence

3.1. ADHESINS

A number of surface polymers or structures of bacteria play roles in their adherence. The presence of fimbriae, in particular, has been implicated (Finlay and Falkow, 1989). Isolates of *E. coli* from pyelonephritis patients can have several adhesins, including type 1, P, S, and F fimbriae (Arthur et al., 1989; Finlay and Falkow, 1989), while Type 1

fimbriae aid in colonization of the catheter (Mobley et al., 1988). Similarly, Type 3 fimbriae, or MR/K hemagglutinin, of *P. stuartii* was positively correlated with long episodes of bacteriuria in catheterized patients and may promote colonization and persistence by adherence to the catheter surface (Mobley et al., 1986, 1988). With *P. aeruginosa*, both fimbriae and extracellular polysaccharide, particularly alginate, have been implicated in its adherence (McEachran and Irvin, 1985). Not only can fimbriae and extracellular polymers bind cells to solid surfaces, but they also can mediate binding among cells (Costerton et al., 1985; Kolenbrander, 1988) or, in the case of some polymers, prevent cell association (Pringle et al., 1983). The potential for interactions among cells, adhesive polymers, and proteins can change in time, as synthesis of surface structures or polymers is initiated, ceased, or modified in response to environmental conditions (Ombaka et al., 1983; Brown and Williams, 1985b; Mulligan and Gibbs, 1989).

In the urinary tract, urease production by catheter-associated bacteria could have a particularly important influence on the biofilm community (Jones and Mobley, 1987; Mobley and Warren, 1987). The result of urea hydrolysis is a rapid rise in pH due to the production of ammonium, which would have a considerable effect on neighboring bacteria in the biofilm. Moreover, the crystallization of struvite and apatite, ultimately leading to stone formation, begins in the biofilm.

3.2. DISSOLVED PROTEINS

Production and presence of proteins can also influence biofilm community characteristics, by influencing adhesive interactions or the physiological success of particular members of the community. Adhesive interactions can be influenced by proteins in two ways. First, interference can occur when proteins bind with adhesins. Tamm-Horsfall protein (THP), for example, apparently binds to Type 1 fimbriae on *E. coli*, reducing the ability of the cells to bind to mannose receptors of the urinary tract epithelium (Orskov et al., 1980). This effect is dependent on concentration, however, as concentrations of >100 *u*g/ml inhibit, but 5-25 *u*g/ml concentrations actually increase adherence (Duncan, 1988). Similarly, THP binds to Type 1 fimbriae of *P. stuartii* (Mobley et al., 1988).

Second, proteins can inhibit adhesion through steric hindrance (Robb, 1984). Dissolved or surface-adsorbed protein, e.g., bovine serum albumin, can prevent bacterial adhesion to solid substrata through steric effects by adsorbing either to the substratum or to the bacterial surfaces (Fletcher, 1976; Fletcher and Marshall, 1982). THP has been shown to bind nonspecifically with bacterial surface polymers (Schachner et al., 1987), and could thus modify the efficiency of bacterial adhesives. Steric hindrance caused by protein binding to substrata has been illustrated by BSA interfering with staphylococcal adhesion to intravascular catheters (Vaudaux et al., 1989) and pseudomonad adhesion to polystyrene (Fletcher, 1976). Proteins can indirectly influence adhesiveness if they are utilized as nutrients and thus induce modifications in cell surface polymers. For example, the synthesis of bacterial surface polysaccharides and fimbriae of Gram-

negative urinary pathogens was considerably influenced by culture medium composition (Chan and Bruce, 1983). For these reasons, THP and other dissolved substances, such as oligosaccharides (Parkkinen et al., 1988), in urine are likely to influence adhesive interactions to some extent.

3.3. SUBSTRATUM PROPERTIES

An environmental factor which can influence bacterial adhesion and surface colonization is the composition of the underlying surface. The numbers of attached bacteria can vary considerably on substrata such as plastics, metals, and glass (Fletcher and Loeb, 1979; Pringle and Fletcher, 1983; van Pelt et al., 1985). Clinical studies have also shown that bacteria may preferentially colonize different materials, such as the frequent occurrence of *Staphylococcus epidermidis* on organic polymer implants, as compared with *S. aureus* which was the major colonizer of metals, bone and joint, and soft tissue (Gristina, 1989). Similarly, the adhesion of *E. coli* and *Klebsiella* was greater to latex and latex-Teflon catheters than to silicone catheters *in vitro* (Sugarman, 1982). The surface properties which can influence adhesion are surface charge (Fletcher and Loeb, 1979), surface free energy (van Pelt et al., 1985; Busscher et al., 1986), hydrophilicity-hydrophobicity (Pringle and Fletcher, 1983, 1986), and texture (Baker, 1984). From the standpoint of preventing adhesion, one of the most promising surface properties is hydration. Hydrogel materials resist both prokaryotic (Pringle and Fletcher, 1986) and eukaryotic (Pekala et al., 1986; Owens et al., 1987) adhesion. The influence of substratum composition on polymicrobial colonization of catheters is relatively unexplored, and there may be potential for reducing colonization by using hydrogel materials.

4. Protection Against Antibiotics

One reason biofilms are particularly significant in the clinical situation is that bacteria in biofilms are less sensitive to antibiotic treatment than free bacteria. For example, *P. aeruginosa* colonizing catheter surfaces were considerably more resistant to tobramycin than free cells (Nickel et al., 1985a,b) or cells resuspended from the catheter biofilm (Anwar et al., 1989). Some biofilm bacteria remained viable at 1000 *ug*/ml tobramycin, while free cells were killed by 50 *ug*/ml (Nickel et al., 1985a). Similarly, coagulase-negative staphylococci colonizing polyvinylchloride catheters (Sheth et al., 1985) or a range of biomaterials (Gristina et al., 1989) were more resistant to a range of antibiotics than were free cells. Gristina et al. (1989) suggested that the degree of resistance was species-dependent and influenced by substratum composition.

A possible reason for this enhanced resistance to antibiotics in biofilms was that the biofilm bacteria were protected by the intercellular polymeric matrix, which retarded diffusion of antibiotic to deeper layers (Costerton et al., 1985). More recent evidence (Gristina et al., 1989; Nichols et al., 1988, 1989) and the application of models (Slack and Nichols, 1982; Sheth et al., 1985; Nichols et al., 1989), however, indicate that although polymers may restrict penetration of antibiotics somewhat, this cannot fully

account for the observed increases in resistance. For example, although tobramycin was bound by the extracellular polysaccharide of *P. aeruginosa*, the resultant decrease in diffusion did not significantly increase penetration time with a spherical microcolony of 125 *u*m radius (Nichols et al., 1988). Other studies have found no relationship between bacterial slime production by coagulase-negative staphylococci and their survival on catheters (Kunin and Steele, 1985) or other biomaterials (Gristina et al., 1989) after antibiotic treatment.

An alternative explanation for the decreased susceptibility of biofilm bacteria is that they are in a different metabolic state than free bacteria. Biofilm bacteria are essentially in the stationary growth phase, whereas free bacteria in the log phase have been used for comparisons (Brown et al., 1988). Therefore, measured differences in susceptibility between free and attached bacteria may be misleading.

Biofilm bacteria are in a microenvironment different from that of free cells, and consequently are probably phenotypically different because of different nutrient or physicochemical conditions, such as osmolarity. There are many examples of differences in metabolic activity (e.g., growth rate, substrate uptake, respiration) between attached and free cells (cf. Fletcher, 1985). Bacterial characteristics which are likely to be influenced by ambient conditions are outer membrane proteins (Griffiths et al, 1983; Brown and Williams, 1985b), such as porins (Lugtenberg and van Alpen, 1983), lipopolysaccharides (Dean et al., 1977), outer membrane phospholipids (Minnikin et al., 1974; Cozens and Brown, 1983), and cation content (Kenward et al., 1979). All of these modifications could theoretically influence antibiotic sensitivity (Brown and Williams, 1985a). For example, changes in porins can alter sensitivities (Lugtenberg and van Alpen, 1983), such as the resistance of *P. aeruginosa* to gentamicin, polymyxin, and EDTA which was correlated with induction of H1 protein (Nicas and Hancock, 1980). Susceptibility to beta-lactam antibiotics is influenced by changes in penicillin-binding proteins in the cytoplasmic membrane (Brown and Williams, 1985b). Reduction in membrane phospholipids and increase in lipopolysaccharide content was correlated with an increased resistance of *P. fluorescens* to polymyxin B (Dorrer and Teuber, 1977). Some antibiotics, e.g., beta-lactams, are dependent upon bacterial growth for their action, and thus slow- or non-growing cells are not susceptible (Tuomanen et al., 1986).

5. Conclusions

The formation of biofilms on surfaces is clearly a serious impediment to the use of both temporary and permanent implants. Once bacteria have colonized an implant surface, not only can this act as a disseminating focus for infection, but can also lead to malfunction of the device and destruction of adjacent tissue. It may be possible to reduce the impact of biofilm formation through selection of biomaterials that are less prone to bacterial adherence and colonization, but this is not yet a workable strategy. Of major concern is the reduced antibiotic susceptibility of biofilm bacteria. It is a serious obstacle in treatment of infections associated with all biomaterials - not just catheter surfaces -

and has important implications for the physiology and ecology of surface-associated organisms.

6. References

Anwar, H., van Biesen, T., Dasgupta, M., Lam, K. and Costerton, J.W. (1989) 'Interaction of biofilm bacteria with antibiotics in a novel *in vitro* chemostat system', Antimicrob. Agents Chemother. 33, 1824-1826.

Arthur, M., Johnson, C.E., Rubin, R.H., Arbeit, R.D., Campanelli, C., Kim, C., Steinbach, S., Agarwal, M., Wilkinson, R., and Goldstein, R. (1989) 'Molecular epidemiology of adhesin and hemolysin virulence factors among uropathogenic *Escherichia coli*', Infect. Immun. 57, 303-313.

Baker, J.H. (1984). Factors affecting the bacterial colonization of various surfaces in a river. Can. J. Microbiol. 30, 511-515.

Brown, M.R.W. and Williams, P. (1985a) 'Influence of substrate limitation and growth phase on sensitivity to antimicrobial agents', J. Antimicrob. Chemother. 15 Suppl. A, 7-14.

Brown, M.R.W. and Williams, P. (1985b) 'The influence of environment on envelope properties affecting survival of bacteria in infections', Ann. Rev. Microbiol. 39, 527-556.

Brown, M.R.W., Allison, D.G. and Gilbert, P. (1988) 'Resistance of bacterial biofilms to antibiotics: a growth related effect?', J. Antimicrob. Chemother. 22, 777-783.

Busscher, H.J., Uyen, M.H.W.J.C., van Pelt, A.W.J., Weerkamp, A.H. and Arends, J. (1986) 'Kinetics of adhesion of the oral bacterium *Streptococcus sanguis* CH3 to polymers with different surface free energies', Appl. Environ. Microbiol. 51, 910-914.

Chan, R.C.Y. and Bruce, A.W. (1983) 'The influence of growth media on the morphology and *in vitro* adherence characteristics of Gram-negative urinary pathogens', J. Urol. 129, 411-417.

Chan, R.C.Y., Reid, G., Irvin, R.T., Bruce, A.W. and Costerton, J.W. (1985) 'Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments', Infect. Immun. 47, 84-89.

Costerton, J.W., Marrie, T.J. and Cheng, K.-J. (1985) 'Phenomena of bacterial adhesion', in D.C. Savage and M. Fletcher (eds.), Bacterial Adhesion, Plenum Press, New York, pp. 3-43.

Cozens, R.M. and Brown, M.R.W. (1983) 'Effect of nutrient depletion on the sensitivity of *Pseudomonas cepacia* to antimicrobial agents', J. Pharmaceu. Sci. 72, 1363-1365.

Dean, A.C.R., Ellwood, D.C., Melling, J. and Robinson, A. (1977) 'The action of antibacterial agents on bacteria grown in continuous culture', in A.C.R. Dean, D.C. Ellwood, C.G.T. Evan and J. Melling (eds), Continuous Culture, vol. 6, Ellis Horwood, Chichester, UK, pp. 251-261.

Dickinson, G.M. and Bisno, A.L. (1989) 'Infections associated with indwelling devices: concepts of pathogenesis; infections associated with intravascular devices', Antimicrob. Agents Chemother. 33, 597-601.

Dorrer, E. and Teuber, M. (1977) 'Induction of polymyxin resistance in *Pseudomonas fluorescens* by phosphate limitation', Arch. Microbiol. 114, 87-89.

Duncan, J.L. (1988) 'Differential effect of Tamm-Horsfall protein on adherence of *Escherichia coli* to transitional epithelial cells', J. Infect. Dis. 158, 1379-1382.

Finlay, B.B. and Falkow, S. (1989) 'Common themes in microbial pathogenicity', Microb. Rev. 53, 210-230.

Fletcher, M. (1976) 'The effects of proteins on bacterial attachment to polystyrene', J. Gen. Microbiol. 94, 400-404.

Fletcher, M. (1985) 'Effect of solid surfaces on the activity of attached bacteria', in D.C. Savage and M. Fletcher (eds.), Bacterial Adhesion, Plenum Press, New York, pp. 339-362.

Fletcher, M. and Loeb, G.I. (1979) 'The influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces', Appl. Environ. Microbiol. 37, 67-72.

Fletcher, M. and Marshall, K.C. (1982) 'Bubble contact angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment', Appl. Environ. Microbiol. 44, 184-192.

Griffiths, E., Stevenson, P. and Joyce, P. (1983) 'Pathogenic *Escherichia coli* express new outer membrane proteins when growing *in vivo*', FEMS Microbiol. Lett. 16, 95-99.

Gristina, A.G. (1989) 'Biomaterial-centered infection: microbial adhesion versus tissue intergration', Science 237, 1588-1595.

Gristina, A.G., Jennings, R.A., Naylor, P.T., Myrvik, Q.N. and Webb, L.X. (1989) 'Comparative *in vitro* antibiotic resistance of surface-colonizing coagulase-negative staphylococci', Antimicrob. Agents Chemother. 33, 813-816.

Jewes, L.A., Gillespie, W.A., Leadbetter, A., Myers, B., Simpson, R.A., Stower, M.J. and Viant, A.C. (1988) 'Bacteriuria and bacteraemia in patients with long-term indwelling catheters - a domiciliary study', J. Med. Microbiol. 26, 61-65.

Jones, B.D. and Mobley, H.L.T. (1987) 'Genetic and biochemical diversity of ureases of *Proteus*, *Providencia*, and *Morganella* species isolated from urinary tract infection', Infect. Immun. 55, 2198-2203.

Kenward, M.A., Brown, M.R.W. and Fryer, J.J. (1979) 'The influence of calcium or magnesium on the resistance to EDTA, polymyxin B or cold shock, and the composition of *Pseudomonas aeruginosa* in glucose or magnesium depleted batch cultures', J. Appl Bacteriol. 47, 489-503.

Kolenbrander, P.E. (1988) 'Intergeneric coaggregation among human oral bacteria and ecology of dental plaque', Annu. Rev. Microbiol. 42, 627-656.

Kunin, C.M. and Steele, C. (1985) 'Culture of the surfaces of urinary catheters to sample urethral flora and study the effect of antimicrobial therapy', J. Clin. Microbiol. 21, 902-908.

Lugtenberg, B. and van Alpen, L. (1983) 'Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria', Biochim. Biophys. Acta 737, 51-115.

McEachran, D.W. and Irvin, R.T. (1985) 'Adhesion of *Pseudomonas aeruginosa* to human buccal epithelial cells: evidence for two classes of receptors', Can. J. Microbiol. 31, 563-569.

McEldowney, S. and Fletcher, M. (1987) 'Adhesion of bacteria from mixed cell suspension to solid surfaces', Arch. Microbiol. 148, 57-62.

McLean, R.J.C., Nickel, J.C., Cheng, K.-J. and Costerton J.W. (1988) 'The ecology and pathogenicity of urease-producing bacteria in the urinary tract', CRC Crit. Rev. Microbiol. 16, 37-79.

Minnikin, D.E., Abdulrahimzadeh, H. and Baddiley, J. (1974) 'Replacement of acidic phospholipids by acidic glycolipids in *Pseudomonas diminuta*', Nature, Lond. 249, 268-269.

Mobley, H.L.T. and Warren, J.W. (1987) 'Urease-positive bacteriuria and obstruction of long-term urinary catheters', J. Clin. Microbiol. 25, 2216-2217.

Mobley, H.L.T., Chippendale, G.R., Tenney, J.H. and Warren, J.W. (1986) 'Adherence to uroepithelial cells of *Providencia stuartii* isolated from the catheterized urinary tract', J. Gen. Microbiol. 132, 2863-2872.

Mobley, H.L.T., Chippendale, G.R., Tenney, J.H., Mayrer, A.R., Crisp, L.J., Penner, J.L. and Warren, J.W. (1988) 'MR/K hemagglutination of *Providencia stuartii* correlates with adherence to catheters and with persistence in catheter-associated bacteriuria', J. Infect. Dis. 157, 264-271.

Mulligan, C.N. and Gibbs, B.F. (1989) 'Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa*', Appl. Environ. Microbiol. 55:3016-3019.

Nicas, T. and Hancock, R.E.W. (1980) 'Outer membrane protein H1 of *Pseudomonas aeruginosa*: involvement in adaptive and mutational resistance to ethylene-diaminetetracetate, polymyxin B, and gentamicin', J. Bacteriol. 50, 715-731.

Nichols, W.W., Dorrington, S.M., Slack, M.P.E. and Walmsley, H.L. (1988) 'Inhibition of tobramycin diffusion by binding to alginate', Antimicrob. Agents Chemother. 32, 518-523.

Nichols, W.W., Evans, M.J., Slack, M.P.E. and Walmsley, H.L. (1989) 'The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*', J. Gen. Microbiol. 135, 1291-1303.

Nickel, J.C., Wright, J.B., Ruseska, I., Marrie, T.J., Whitfield, C. and Costerton, J.W. (1985a) 'Antibiotic resistance of *Pseudomonas aeruginosa* colonizing a urinary catheter *in vitro*', Eur. J. Clin. Microbiol. 4, 213-218.

Nickel, J.C., Ruseska, I., Wright, J.B. and Costerton, J.W. (1985b) 'Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material', Antimicrob. Agents Chemother. 27, 619-624.

Ombaka, E.A., Cozens, R.M. and Brown, M.R.W. (1983) 'Influence of nutrient limitation of growth and stability and production of virulence factors of mucoid and

nonmucoid strains of *Pseudomonas aeruginosa*', Rev. Infect. Dis. Suppl. 5, S880-S888.

Orskov, I., Ferencz, A and Orskov, F. (1980) 'Tamm-Horsfall protein or uromucoid is the normal urinary slime that traps type 1 fimbriated *Escherichia coli*' (Abstr.) Lancet ii, 887.

Owens, N.F., Gingell, D. and Rutter, P.R. (1987) 'Inhibition of cell adhesion by a synthetic polymer adsorbed to glass shown under defined hydrodynamic stress', J. Cell Sci. 87, 667-675.

Parkkinen, J., Virkola, R. and Korhonen, T.K. (1988) 'Identification of factors in human urine that inhibit the binding of *Escherichia coli* adhesins', Infect. Immun. 56, 2623-2630.

Pekala, R.W., Merrill, E.W., Lindon, J., Kushner, L. and Salzman, E.W. (1986) 'Fibrinogen adsorption and platelet adhesion at the surface of modified polypropylene glycol/polysiloxane networks', Biomater. 7, 379-385.

Pringle, J.H. and Fletcher, M. (1983) 'Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces', Appl. Environ. Microbiol. 45, 811-817.

Pringle, J.H. and Fletcher, M. (1986) 'Influence of substratum hydration and adsorbed macromolecules on bacterial attachment to surfaces', Appl. Environ. Microbiol. 51, 1321-1325.

Pringle, J.H., Fletcher, M. and Ellwood, D.C. (1983) 'Selection of attachment mutants during the continuous culture of *Pseudomonas fluorescens* and relationship between attachment ability and surface composition', J. Gen. Microbiol. 129, 2557-2569.

Robb, I.D. (1984) 'Stereo-biochemistry and function of polymers', in K.C. Marshall (ed.), Microbial Adhesion and Aggregation, Springer-Verlag, Berlin, pp. 39-49.

Schachner, M.S., Miniter, P.M., Mayrer, A.R. and Andriole, V.T. (1987) 'Interaction of Tamm-Horsfall protein with bacterial extracts', Kidney Interntl. 31, 77-84.

Sheth, N.K., Franson,T.R. and Sohnle, P.G. (1985) 'Influence of bacterial adherence to intravascular catheters on *in-vitro* antibiotic susceptibility', ii, 1266-1268.

Slack, M.P.E. and Nichols, W.W. (1982) 'Antibiotic penetration through bacterial capsules and exopolysaccharides', J. Antimicrob. Chemother. 10, 368-371.

Stickler, D.J., Harber, M.J. and Chawla, J.C. (1988) '*Pseudomonas aeruginosa* and long-term indwelling bladder catheters', Arch. Phys. Med. Rehabil. 69, 25-28.

Sugarman, B. (1982) 'Adherence of bacteria to urinary catheters', Urol. Res. 10, 37-40.

Tenney, J.H. and Warren, J.W. (1988) 'Bacteriuria in women with long-term catheters: paired comparison of indwelling and replacement catheters', J. Infect. Dis. 157, 199-202.

Tuomanen, E., Cozens, R., Tosch, W., Zak, O. and Tomasz, A. (1986) 'The rate of killing of *Escherichia coli* by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth', J. Gen. Microbiol. 132, 1297-1304.

van Pelt, A.W.J., Weerkamp, A.H., Uyen, M.H.W.J.C., Busscher, H.J., de Jong, H.P. and Arends, J. (1985) 'Adhesion of *Streptococcus sanguis* CH3 to polymers with different surface free energies', Appl. Environ. Microbiol. 49, 1270-1275.

Vaudaux, P., Pittet, D., Haeberli, A., Huggler, E., Nydegger, U.E., Lew, D.P. and Waldvogel, F.A. (1989) 'Host factors selectively increase staphylococcal adherence on inserted catheters: a role for fibronectin and fibrinogen or fibrin', J. Infect. Dis. 160, 865-875.

Warren, J.W. (1986) '*Providencia stuartii*: a common cause of antibiotic-resistant bacteriuria in patients with long-term indwelling catheters', Rev. Infect. Dis. 8, 61-67.

Warren, J.W., Damron, D., Tenney, J.H., Hoopes, J.M., Deforge, B. and Muncie, H.L. Jr. (1987) 'Fever, bacteremia, and death as complications of bacteriuria in women with long-term urethral catheters', J. Infect. Dis. 155, 1151-1158.

BIOMATERIALS BIOCOMPATIBILITY IN HEALTH PROBLEMS

H.J. BUSSCHER[1], J.M. SCHAKENRAAD[2] and H.C. VAN DER MEI[1]
Materia Technica[1] and Biomedical Technology Centre[2]
University of Groningen
Antonius Deusinglaan 1
9713 AV Groningen, the Netherlands

1. Biocompatibility Requirements of Biomaterials

According to the book "Definitions in Biomaterials" by D.F. Williams (1987), the title of this chapter "Biomaterials Biocompatibility in Health Problems" can alternatively be formulated as:
The requirements set to a "non-viable material used in a medical device, intended to interact with biological systems" in order to "perform with an appropriate host response in a specific application.

There is an increasing use of biomaterials in the human body (Silver and Doillon, 1989; Black, 1983; Sauvage *et al.*,1987; Williams, 1981; Pilliar, 1987) as cardiovascular implants (heart valves, pacemakers, vascular grafts), dental implants, plastic and reconstructive implants (breast, nose, chin, abdominal wall and other prostheses), ophthalmic implants (lenses, retinal surgery), orthopaedic prostheses, neurosurgical implants and extracorporeal devices (oxygenators, catheters). All these implants interact with different elements of the biological system in which they are supposed to perform. Hence the word "biocompatibility" refers to different primary objectives for the various applications. In case of a vascular graft, the primary objective is to create non-thrombogenicity; for a dental implant osseointegration is a "conditio sine qua non", abdominal wall patches should not demonstrate adhesion of the bowel to the visceral side, but adhesion of connective tissue cells on the parietal side is necessary.

A secondary objective for all applications of biomaterials in the human body is to show minimal adhesion of infectious microorganisms. At present, virtually all biomaterials implanted in the human body become infected sooner or later (Gristina, 1987). Since the infectious microorganisms usually appear protected in a biofilm by exopolysaccharides (the "glycocalyx"), they are resistant to antibiotics and persist until the "foreign-body" is removed (Nickel *et al.*, 1985a).

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 309–326.

There are various approaches that can be chosen in order to enhance the biocompatibility of biomaterials. Chemical modifications of biomaterials are aimed at modifying those properties, influencing their interactions with cells, bacteria, platelets or proteins. These properties include amongst others wettability (surface free energy) and charge, as corollaries of the chemical surface composition and roughness. Sometimes these modifications yield completely inert surfaces showing minimal "bioadhesion", whereas other times maximal adhesion results as the required goal. In the latter case, the approach is aimed at enhancing the adhesion of natural tissue to the biomaterial, which is in many situations an alternative to prevent adhesion of platelets or infectious microorganisms.

In this chapter, we will discuss the basics of the interactions of "bioparticles" with biomaterials in a physiologic environment, using selected examples illustrating the various primary and secondary objectives of the word "biocompatibility".

2. Chemical Modifications of Biomaterials Surfaces

Physico-chemical properties of biomaterials surfaces have a major influence on their ultimate biocompatibility. The influence of the biomaterials surface is either a direct, or an indirect one in case the biomaterial is covered with a proteinaceous "conditioning film". Chemical modification of biomaterials surfaces is usually done by glow-discharge treatment, ion etching or wet chemistry, therewith affecting the wettability, charge properties and roughness of the surfaces. In the next three sections, examples will be discussed from different biomedical areas to demonstrate the effects of the above surface properties on the biocompatibility of biomaterials.

2.1. INTERACTION OF ESCHERICHIA COLI WITH CATHETER SURFACES

Urinary catheters are amongst the most used (temporary) implants in hospitalized patients. On an average 10-27% of all catheterized hospital patients acquire an urinary catheter-associated infection within 5 days after catheterization (Nickel *et al.*, 1985b). Most frequently, *E. coli* derived from the patients gastrointestinal tract is implicated, although it is not clear how the organism gains entry into the bladder. Due to the increasing use of closed drainage systems, the inter lumen of the catheter can be excluded nowadays as a possible pathway. Most likely, bacteria adhere to the catheter surface upon insertion of the catheter into the urinary tract (especially the far downstream part of the urinary tract may not be regarded as a sterile environment) and thus

gain entry into the bladder where they subsequently grow and form a biofilm (Harkes, 1991). It has also been suggested that bacteria adhere to the outer catheter surface and then migrate over the surface and along the peri-urethral epithelial into the bladder.

Recently, Harkes *et al.* (1991, 1992) studied adhesion, growth and migration of *E. coli* strains on a series of polymethylmethacrylates differing in charge and hydrophobicity. Both their cells as well as their substrata were characterized by contact angle and zeta potential measurements. They carried out their adhesion experiments in a perfusion chamber under laminar flow (1 ml/sec, reported Reynolds number 1.5). Fig. 1 shows strain dependent relations between the numbers of adhering cells and zeta potentials (Fig. 1a) and wettability (Fig. 1b).

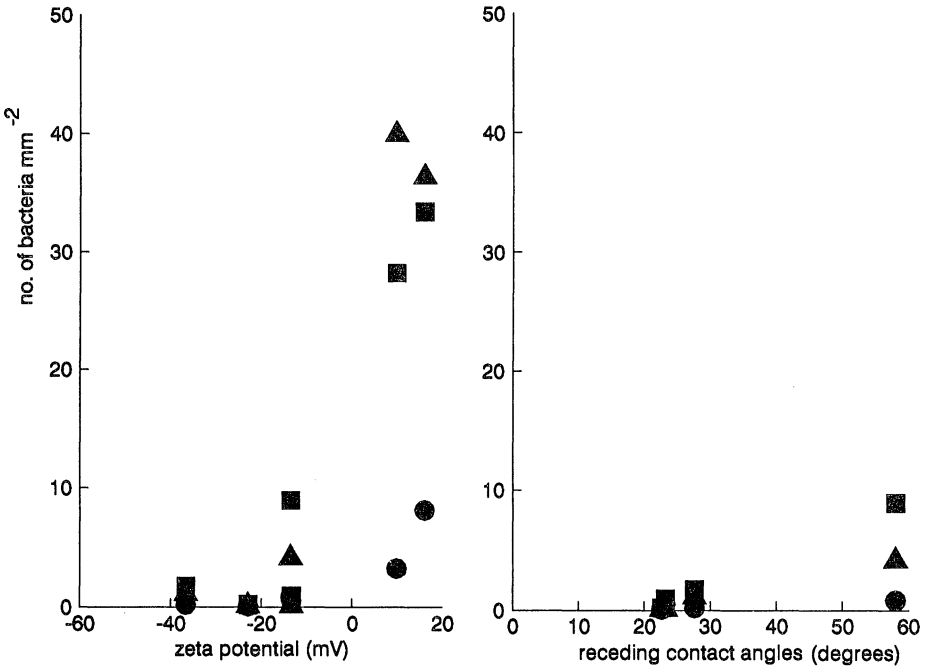


Figure 1. Adhesion, growth and migration of urogenital *E. coli* strains to a series of polymethylmethacrylates with different charge and wettability (adapted from Harkes *et al.*, 1991, 1992).

Figure 1a. Stationary state adhesion values vs. substratum zeta potentials. ● *E. coli* O11K58, ζ = -3 mV; ▲ *E. coli* O157K-, ζ = -9 mV; ■ *E. coli* O161K-; ζ = -34 mV (left).

Figure 1b. Stationary state adhesion values vs. substratum receding contact angles (water). ● *E. coli* O111K58, θ_{water} = 29 degrees; ▲ *E. coli* O157K-; θ_{water} = 18 degrees; ■ *E. coli* O161K-, θ_{water} = 25 degrees (right)

Clearly, adhesion can be discouraged by increasing the negative charge and wettability of the biomaterials. In the same system (Harkes *et al.*, 1991, 1992) growth of *E. coli* on a similar series of polymers was studied, by first adhering bacteria to a density of 5.10^4 cm^{-2} and subsequently flowing with broth. In these experiments, the catheter-associated strains grew more rapidly on surfaces with a relatively high negative zeta potential and low water contact angle than on surfaces with a relatively low negative zeta potential and high water contact angle (see Figs. 1c and 1d). Thus these experiments demonstrate that bacterial adhesion and growth are inversely influenced by physico-chemical substratum properties.

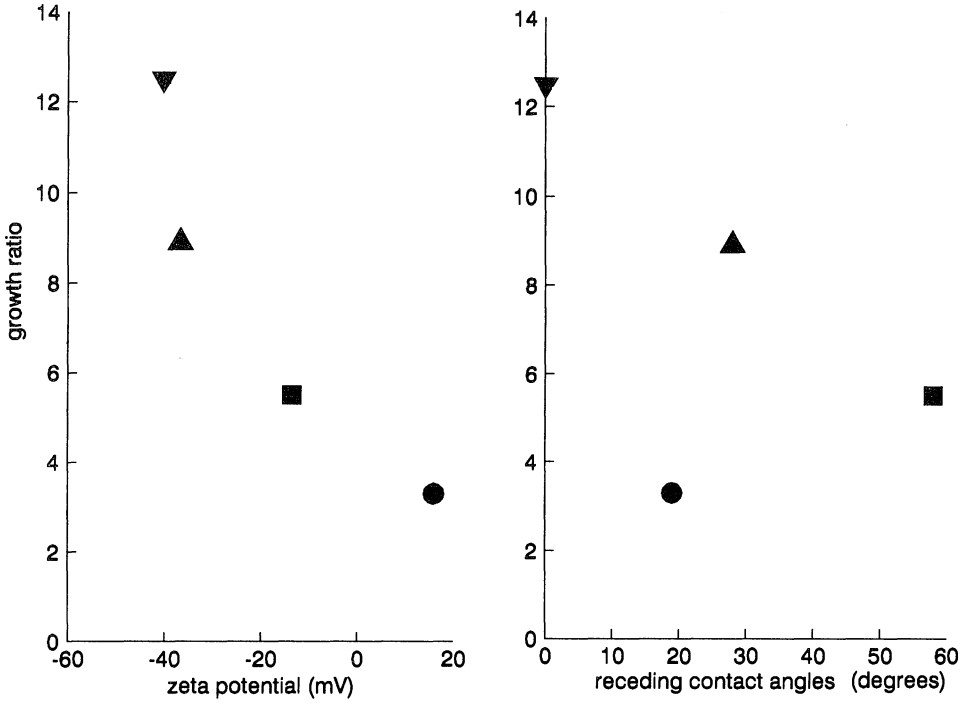


Figure 1. Adhesion, growth and migration of urogenital *E. coli* strains to a series of polymethylmethacrylates with different charge and wettability (adapted from Harkes *et al.*, 1991, 1992).

Figure 1c. Growth ratios after 150 min vs. substratum zeta potentials of *E. coli* O2K2 (ζ = -53 mV). ● PMMA/TMAEMA-Cl; ■ PMMA; ▲ PMMA/MMA; ▼ glass (left).

Figure 1d. Growth ratios after 150 min vs. substratum zeta potentials of *E. coli* O2K2 (Θ_{water} = 57°). For symbols, see Fig. 1c (right).

In addition to their ability to adhere, adherent bacteria may be able to slide over a substratum surface. On perfectly smooth and homogeneous surfaces, "adhesion" and "sliding" should in principle be unrelated phenomena, because adhesion requires forces perpendicular to the substratum surface, whereas migration involves forces parallel to the surface. (Busscher *et al.*, 1990).

Harkes *et al.* (1991, 1992) designed a device to mimic the extraluminal space between an inserted catheter and the epithelial cell lining to study migration of cells over a biomaterial. Their results (see Figs. 1e and 1f) showed that the most hydrophobic and negatively charged cells were the least immobilized on glass, but didnot show any variation in immobilization of the fastest migrating strain on materials with different hydrophobicity and charge.

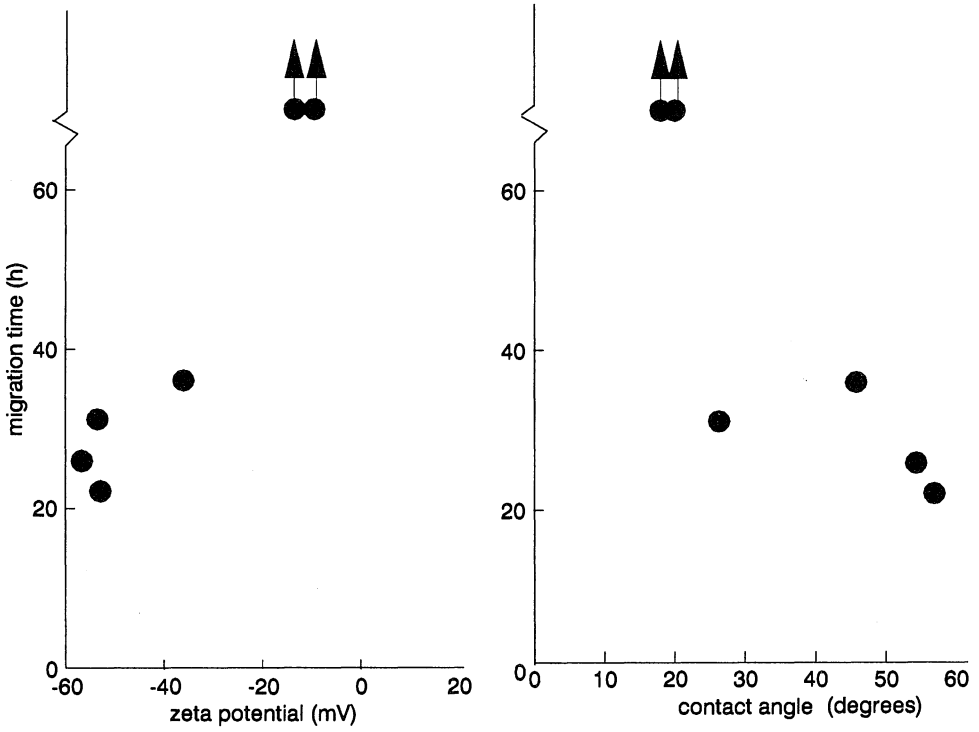


Figure 1. Adhesion, growth and migration of urogenital *E. coli* strains to a series of polymethylmethacrylates with different charge and wettability (adapted from Harkes *et al.*, 1991, 1992).

Figure 1e. Immobilization (1/migration time) of different strains on glass vs. the bacterial zeta potentials (left).

Figure 1f. Immobilization (l/migration time) of different strains on glass vs. the bacterial receding water contact angles (right).

Summarizing, this section shows that chemical modification of biomaterials surfaces may strongly influence the adhesion and growth of microorganisms and therewith the biocompatibility of implants.

2.2. AN IN VITRO AND IN VIVO CASE STUDY ON MODIFIED TEFLON SURFACES

Teflon is a frequently used biomaterial with a broad field of application, ranging from vascular grafts, eardrum-tubes, periodontal membranes to abdominal wall patches. The mechanical properties of Teflon are often the reason to choose this material for a specific application: Teflon is strong, flexible, microporous (e-PTFE; expanded polytetrafluoroethylene) and it is highly bioinert (Gore, 1976). Upon implantation, it evokes a minor foreign body reaction resulting in encapsulation by fibrous tissue (Neel, 1983). Teflon is an extremely hydrophobic material (water contact angle 109°, Schakenraad *et al.*, 1986) and therefore its application is most often in situations where no or minor adhesion to body tissues is needed (Cuadros, 1984; Lamb *et al.*, 1983; Gottlow *et al.*, 1984). When Teflon is applied in situations where good interaction with body tissue is needed (e.g. abdominal wall patches), failure is likely to occur (Jenkins *et al.*, 1983).

It is a challenge to try and combine the optimal mechanical properties of Teflon with surface properties needed in specific clinical applications, i.e. sometimes very hydrophobic (e.g. as vascular prostheses) and other times hydrophilic (e.g. for abdominal wall patches on the parietal side). For that purpose we developed so-called superhydrophobic and hydrophilic Teflon variants. Hydrophobic modification consisted of a 5 hour argon ion beam treatment, followed by oxygen glow discharge for 5 minutes. The entire procedure is described in detail by Busscher *et al.* (1992). The hydrophilic modification consisted of a much milder 45 minutes argon ion beam treatment.
Table 1 summarizes the contact angles of water and α-bromonaphthalene on these modified materials and shows that the hydrophobic modification indeed results in a very high water contact angle, whereas the hydrophilic modification results in a water contact angle comparably low as found on glass. Also significant differences in the contact angle with α-bromonaphthalene, an a-polar probing liquid, were observed.

The superhydrophobicity created, is not only due to a chemical effect, but also to the mechanical effects of the ion etching. A scanning electron micrograph (Fig. 2) of the superhydrophobic Teflon surface illustrates a characteristic roughening with hair-like structures of approximately 40 nm in diameter covering the Teflon surface. This micro-roughening, in combination with the chemical effect of the glow-discharge treatment might be responsible for the superhydrophobicity observed.

TABLE 1. Advancing type contact angles of water and α-bromonaphthalene (sessile droplets) on variously treated Teflon (Fluoroethyelenpropylene, FEP) surfaces.

Material	Water contact angle [degrees]	α-bromonaphthalene contact angle [degrees]
FEP-Teflon	109	73
Hydrophobized FEP-Teflon	>140	97
Hydrophilized FEP-Teflon	6	16

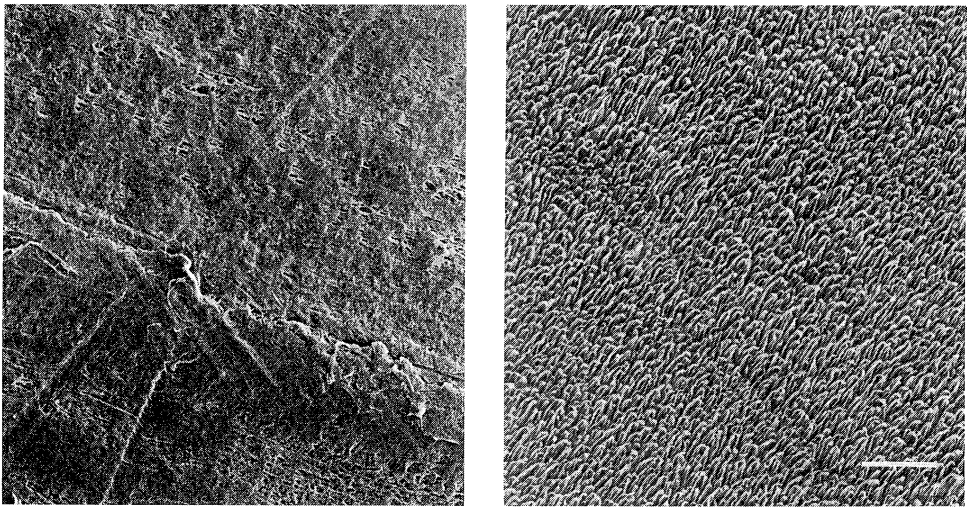


Figure 2. Scanning electron micrographs of untreated FEP-Teflon (left) and hydrophobized FEP-Teflon (right), showing hair-like structures on the surface. The bar indicates 9 μm.

However, there was also a significant effect of the treatment on the adhesion and spreading of human fibroblasts on the various Teflon surfaces *in vitro* (Busscher *et al.*, 1991). After allowing the cells to adhere and spread for 120 minutes, photographs were taken of the spreaded cells and the number of adhered cells per unit area as well as the cell spreading area per material were determined by morphometric image analysis.

The results clearly demonstrate that cells on standard FEP-Teflon adhered and spread considerably less (mean area per cell 209 μm^2) as compared to tissue culture polystrene (control material, 270 μm^2). Cells on the superhydrophobic Teflon showed an even smaller spreading area (158 μm^2), while cell spreading on the hydrophilic Teflon was similar (257 μm^2) to the spreading observed on tissue culture polystyrene. Light micrographs, illustrating these data, are shown in Fig 3.

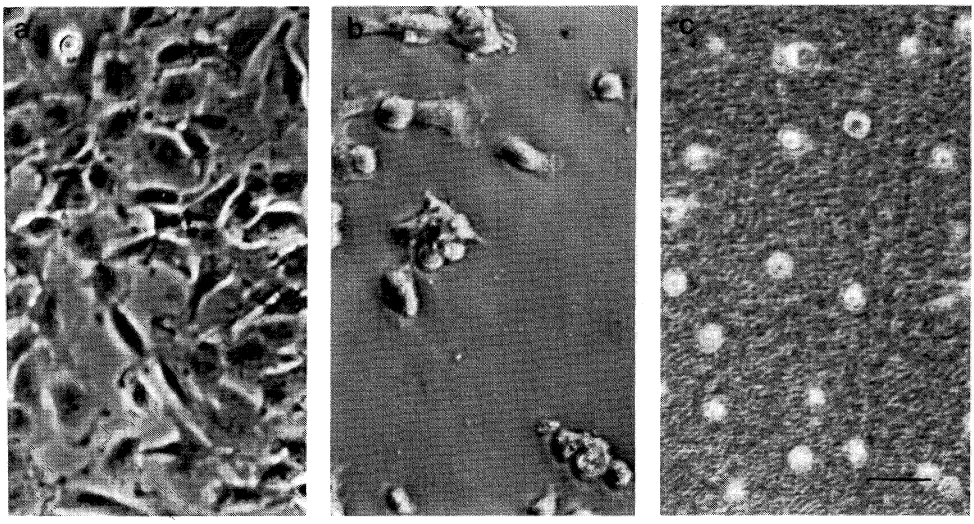


Figure 3. Light micrographs of human skin fibroblasts adhering and spreading on (a) Tissue Culture Polystyrene, (b) untreated FEP-Teflon, (c) hydrophobized FEP-Teflon. The bar indicates 10 μm.

Application of the superhydrophobic Teflon variant in the field of cardiovascular biomaterials is apparent. As already mentioned, e-PTFE has been successfully applied as a vascular graft (diameter > 4 mm), due to its strength, flexibility and bioinertness. In addition, its hydrophobicity creates a minimal interaction surface (Soyer *et al.*, 1972; Graham and Bergan, 1982; Campbell *et al.*, 1979) and patent grafts, both in experimental and clinical situations, over longer periods of time. Complete neoendothelialization was never achieved, however. Moreover, clinical and experimental use of small calibre blood vessels with e-PTFE (1.0 - 1.5 mm internal diameter) has not yet been successful. Several authors report patency up to 40 days of 1 to 1.5 mm internal diameter e-PTFE grafts in the rat or rabbit femoral artery (Watanabe, 1979; Derman and Reichman, 1979). Also in dogs and sheep (carotid) patency up to 10 months was observed, using 3 to 4 mm grafts (Volder and Kolff, 1974; Campbell *et al.*, 1974).

The rabbit carotid artery model is a much more critical model from the thrombogenic point of view, and more resembling the human model, in which early clotting is always observed (Van der Lei *et al.*, 1989; Brennwald and Aebi, 1985; Campbell *et al.*, 1979) particularly for small diameter grafts.

As a first attempt to explore the possible applications of our superhydrophobic Teflon, we set out to prove that our material could successfully be used as a small calibre vascular grafts in the carotid artery of the rabbit (Schakenraad *et al.*, 1992). To this end, commercial e-PTFE vascular grafts, internal diameter 1.5 mm, pore-size 30 μm (Gore, The Netherlands) with a length of approximately 1.0 cm were cut open longitudinally to make the luminal side superhydrophobic, as described before (Busscher *et al.*, 1992). Scanning electron microscopy (Fig. 4) shows that ion-etching and glow-discharge treatment of the Gore-tex vascular graft yields hair-like structures with diameters of several hundred nanometers on the nodes, which are absent on the non-treated Gore-tex grafts (Fig. 4). Following the treatment, the superhydrophobic grafts were closed longitudinally with a running suture and implanted in the left carotid artery of rabbits while refraining from any anti-thrombotic regime. Untreated Gore-tex grafts were implanted in the right carotid artery. Two hours after the insertion of the grafts, the unmodified Gore-tex prosthesis was occluded, while the superhydrophobicly modified graft was still patent. After 1 week of implantation, the rabbits were

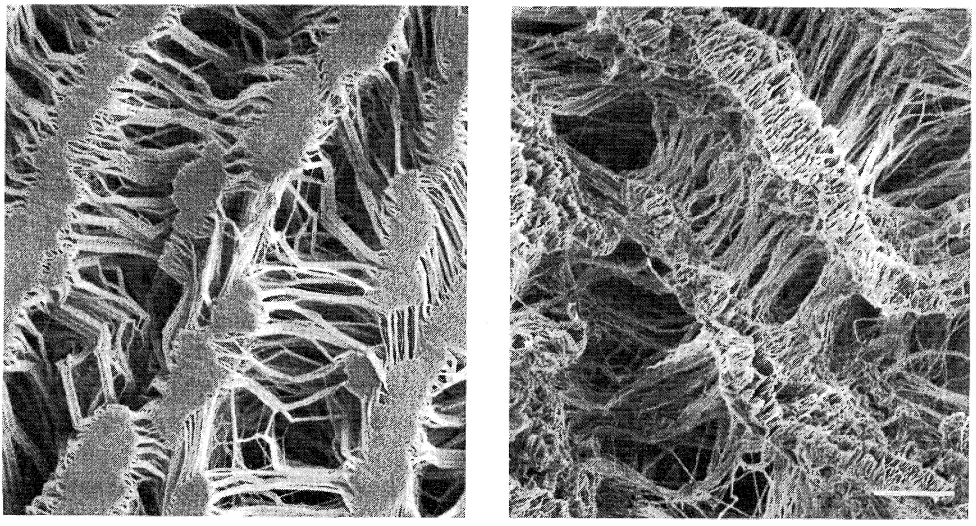


Figure 4. Scanning electron micrographs of the luminal surface of untreated (left) and superhydrophobicly modified e-PTFE Gore-tex vascular prostheses (right, inner diameter: 1.5 mm). Note the hair-like structures on the nodes of the modified Gore-tex. The bar denotes 9 μm.

sacrificed and the grafts were removed using a perfusion-fixation technique, which allows fixation (2% glutaraldehyde) of the graft *in situ*. Before removal, the patency of the grafts was checked once again. The unmodified grafts were indeed occluded, while the treated grafts were patent. Scanning electron micrographs of the luminal surface (Fig. 5) revealed no massive thrombus in the superhydrophobicly modified grafts. Instead, the graft surface was covered with a

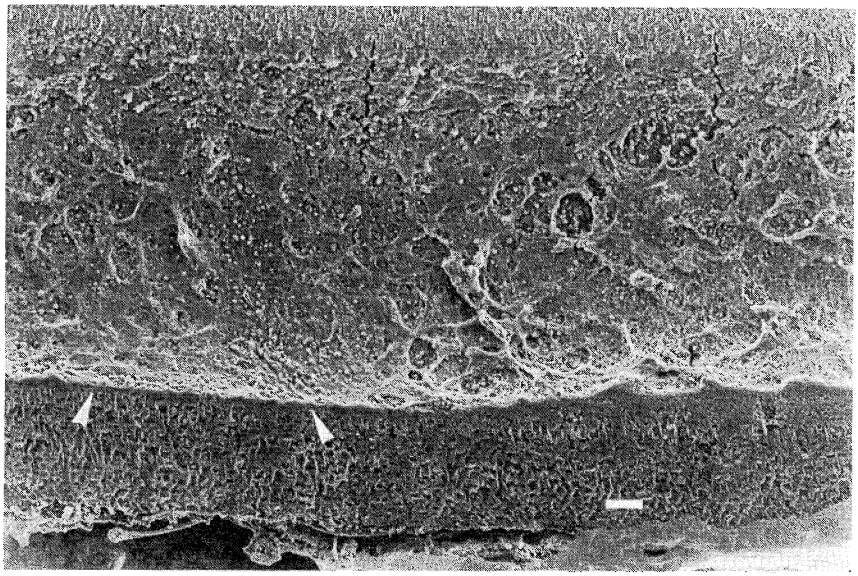


Figure 5. Scanning electron micrograph showing the luminal surface of the superhydrophobic Gore-tex prosthesis after 1 week of implantation. Note the very thin proteinaceous clot layer. The bar denotes 100 μm.

thin layer of proteinaceous material in which platelets were mechanically entrapped. Some monocytes were observed at the surface of the thin adsorbed layer. No leucocytes were observed on or in the adsorbed layer. As judged by their shape and cellular contents (Fig. 6), the platelets at the surface were not activated. At the anastomotic sides, some endothelial overgrowth could be observed (Fig. 7). The thin adsorbed proteinaceous layer is apparently a good matrix for endothelial cell growth and adhesion. Endothelial ingrowth was observed over the first 0.5 - 1.0 mm of the graft lumen. Although the hemodynamic circumstances at the anastomosis are far from optimal, no clot formation nor intimal hyperplasia could be observed. Assuming that endothelial cell growth would continue at a speed of 0.5-1.0 mm per week, as during the first week, a 1 cm vascular graft would be re-endothelialized within 5 to 10 weeks.

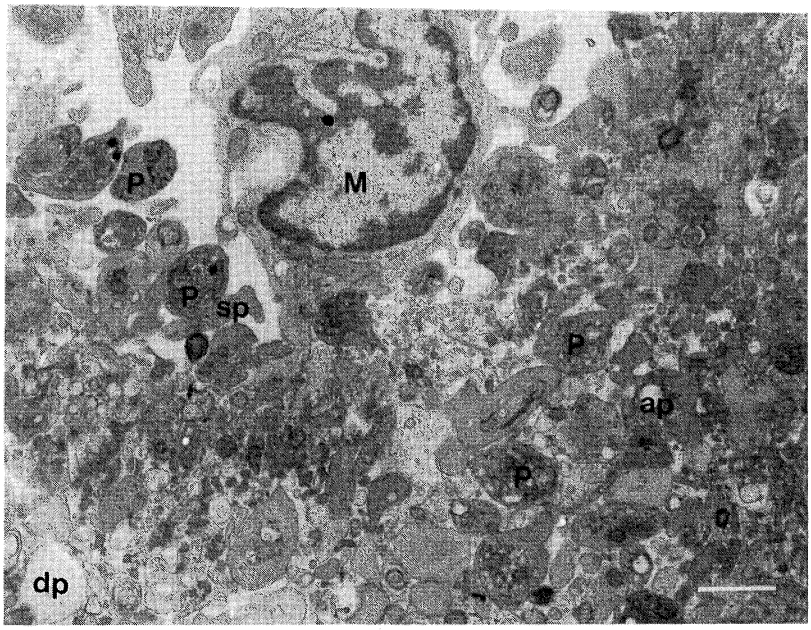


Figure 6. Transmission electron micrograph of a superhydrophobic e-PTFE Gore-tex prosthesis after 1 week implantation in the carotid arter of a rabbit, showing platelets (P) and a monocyte (M) in a granular network. Most platelets are neither activated (ap) nor spread (sp) and their granular contents is not released. Some platelets appear semi-spread and have partially released their contents. Also dead platelets (dp) can be observed. The bar represents 2 μm.

Endothelial coverage of PTFE vascular prostheses was also studied by Campbell *et al.* (1979) who demonstrated a maximum ingrowth of only 0.2 mm per week (rabbit carotid artery). Other animal models/vessel models proved even worse.

At present, we are not sure about the mechanism of passification of this new material. Albumin is known to yield a fully passified surface and is difficult to displace by other plasma proteins from hydrophobic surfaces. Possibly, our surfaces bind large amounts of albumin, either by adsorption or by mechanical retention in the micro-structure created by the treatment. Alternatively, the superhydrophobic surface may exert extremely small interaction forces on the platelets. It is also known that the microstructure at the luminal surface may improve the rheology of blood flow through the prosthesis by providing a stagnant layer at the interface. However, the study shows that the biocompatibility of FEP-Teflon can be greatly improved by the proposed treatment and other biomedical applications, like the use as an abdominal wall patch, are currently under investigation.

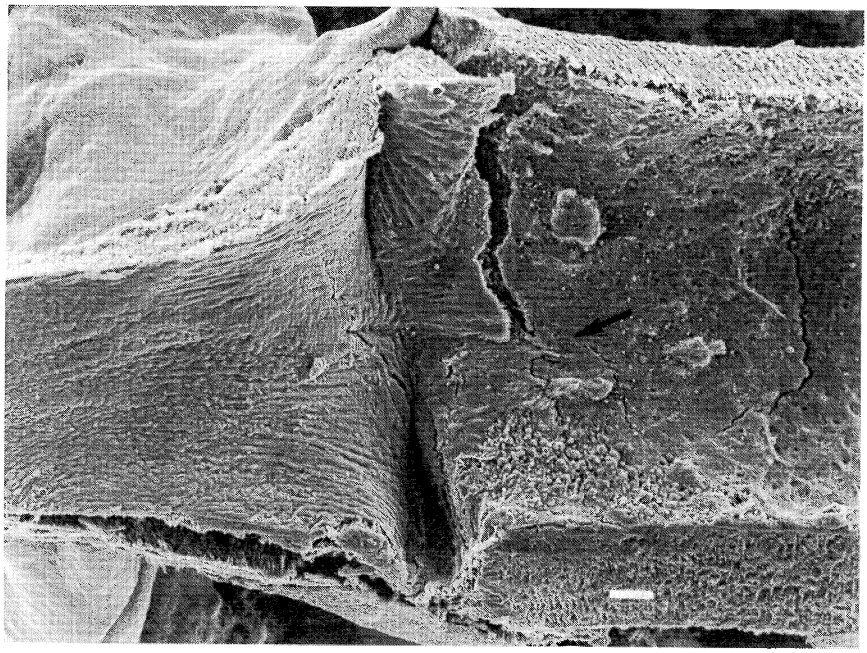


Figure 7. Scanning electron micrograph of the anastomosis between the superhydrophobic e-PTFE Gore-tex prosthesis (right side) and the original artery (left side), showing the endothelial overgrowth (see arrow) from the artery over the prosthesis. The bar denotes 100 μm.

3. Cell-seeding to improve the Biocompatiblity of Biomaterials

Cell-seeding on biomaterials is an approach based on the philosophy that the most biocompatible surface is the natural tissue itself. When applying cell-seeding, the implant is covered with tissue cells so that the implant surface mimics the natural tissue. A frequently given example is the seeding of an endothelial cell lining on artificial vascular grafts to prevent clot formation. Of course the requirements set to the biomaterials surfaces used in combination with cell-seeding techniques are completely opposite to those discussed for the superhydrophobic, completely non-adhesive artificial vascular graft and biomaterials used for cell-seeding should optimally interact with cells.

Also with regard to prevention of microbial infections, cell-seeding might be useful. Gristina (1987) described the ultimate fate of a biomaterials implant as a "race for the surface" between bacteria and tissue cells. If the tissue cells arrive first at the biomaterials surface and a secure bond is formed, long term patency is warranted, although sometimes biomaterials implants are called a microbial time bomb.

Cells seeded on a biomaterial do not only have to adhere and spread well but also have to withstand occasionally high shear forces as e.g. in a vascular graft. This requires different experimental evaluation of cell-biomaterial interactions than used hitherto: Static experiments, in which only the number of adhering cells and their spreaded areas are measured are not sufficient to this end, due to the absence of shear. Van Kooten *et al.* (1992a) recently described the use of a parallel plate flow chamber with *in situ* observation and automated image analysis to observe the response of fibroblasts on different biomaterials when exposed to an incrementally loaded shear. Cells detached at significantly lower shear stresses from a hydrophobic material than from a hydrophilic material (Van Kooten *et al.*, 1992b), whereas furthermore it appeared that cells tended to round up their shape as a response to increasing shear (see Fig. 8 for a summary of results).

A good example of the application of cell-seeding is the vascular prosthesis. Most of the times a vascular prosthesis solely serves as a means of blood transport, however, the endothelial recovery on the luminal side of the vascular prosthesis is often incomplete if not absent, though highly desirable. Endothelial tissue will reduce the thrombogenicity of the vascular graft by e.g. the production of prostacyclin, a potent inhibitor of platelet aggregation. Spontaneous re-endothelialization of vascular grafts is a slow and limited process, because mostly, endothelial cells only originate from the anastomosis of the host blood vessel. Successful and rapid re-endothelialization can be achieved using endothelial cell-seeding techniques.

Two different approaches are commonly applied: direct harvesting of endothelial cells (Herring *et al.*, 1978; Graham *et al.*, 1980a) from host tissue (mostly the saphenous vein), or pre-culturing of endothelial cells and seeding at the time of operation (Graham *et al.*, 1980b). Briefly, the endothelial cells, cultured or directly harvested, are suspended in an aliquot of host blood. This suspension is used to pre-clot the luminal side of the vascular prosthesis, prior to implantation. The seeded endothelial cells will soon afterwards cover the luminal side of the graft, thus improving its anti-thrombogenicity.

However, as a complication, the freshly seeded endothelial cells are often removed, due to the high shear forces of arterial blood. This process can possibly be reduced or prevented by offering the endothelial cell an optimal matrix of smooth muscle cells. Smooth muscle cells form the neomedia in an artery and normaly support and form the achoring matrix for the luminal endothelial cells. Experimental work on smooth muscle cell seeding proved this hypothesis (Yue *et al.*, 1988; Van Oene *et al.*, 1987).

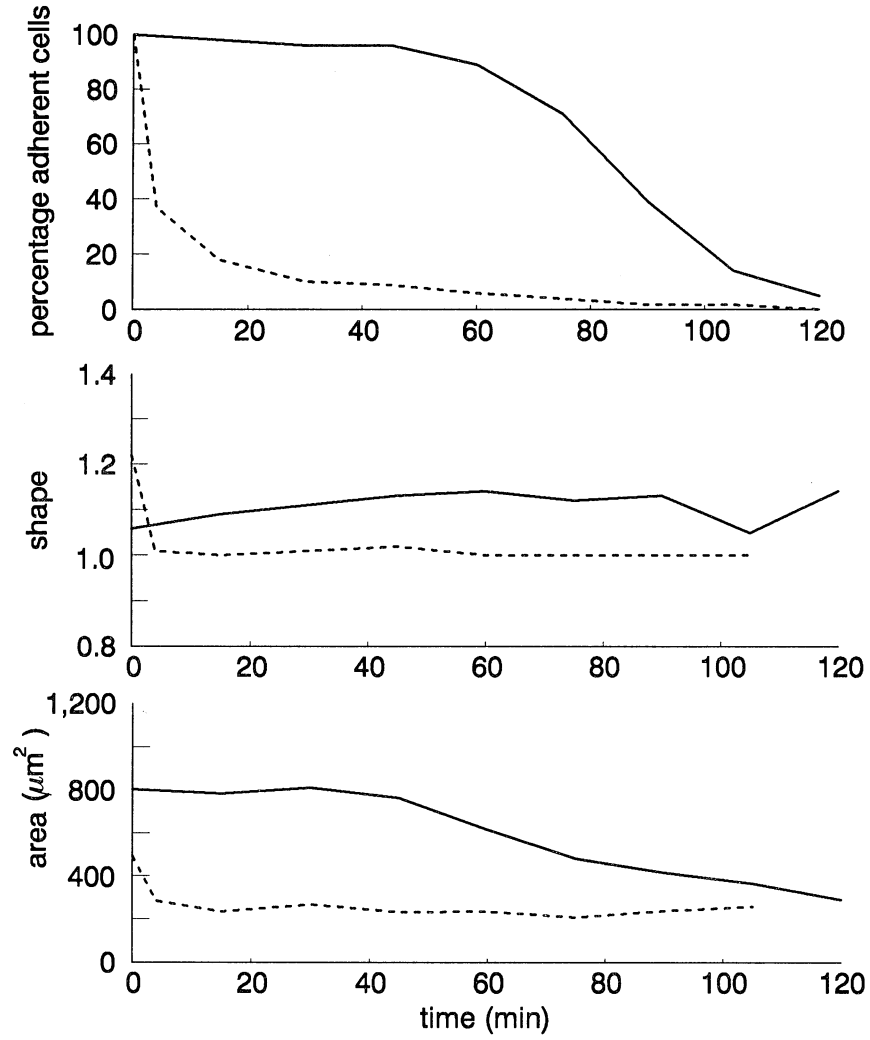


Figure 8. Behaviour of human skin fibroblasts on a hydrophobic (dashed lines) and a hydrophilic (solid lines) material as a response to an incrementally loaded fluid flow (flow was increased every 15 min by 3 ml/s) through a parallel plate flow chamber (chamber height 0.02 cm):
- percentage of cells present expressed relative to the initial number of cells
- circular projected shape (1 indicates a circle)
- area of the spread cells
(adapted from Van Kooten *et al.*, 1992b)

An alternative approach is represented by recently published work by Sedlarik *et al.* (1990). They aimed at providing a very rapid re-endothelialization by covering the vascular prosthesis with meshes of vascular tissue. This tissue could be obtained during the operation, from the operation site. A very small piece of arterial or venous tissue (0.2 - 0.3 mm) could cover a vascular prosthesis of 1-1.5 cm. Sedlarik *et al.* (1990) demonstrated that endothelial cells not only migrate through the porous vascular prosthesis towards the lumen of the vascular graft to form a neointima, but also that smooth muscle cells migrated to form a neomedia. Within one week the authors found a continuous endothelial layer formed on the luminal side of the vascular prosthesis as well as a neomedia with already circularly oriented smooth muscle cells: the endothelial cells on the luminal side did not wash away in the bloodstream since the necessary matrix (provided by the smooth muscle cells) was present.

4. Summary

In summary, we have shown that adhesion, growth and migration of microorganisms, cellular adhesion and spreading as well as the blood compatibility of biomaterials can be greatly influenced by modifying the surface properties of the biomaterial under study.

5. References

Black, M.M., Drury, P.J. and Tindale, W.B. (1983) 'Twenty-five years of heart valve substitutes: A review. J. R. Soc. Med. 76, 667.

Brennwald, J. and Aebi, M. (1985) 'Experimental studies on artificial prostheses in microvascular surgery', Handchir. Microchir. Plast. chir. 1, 41-42.

Busscher, H.J., Sjollema, J. and Van der Mei, H.C. (1990) 'Relative importance of surface free energy as a measure of hydrophobicity in bacterial adhesion to solids', in R.J. Doyle and M. Rosenberg (eds.), Microbial Cell Surface Hydrophobicity, American Society for Microbiology, Washington, pp. 335-359.

Busscher, H.J., Stokroos, I., Golverdingen, J.G. and Schakenraad, J.M. (1991) 'Adhesion and spreading of human fibroblasts on superhydrophobic FEP-Teflon', Cells and Materials 1, 243-250.

Busscher, H.J., Stokroos, I., Van der Mei, H.C., Rouxhet P.G. and Schakenraad, J.M. (1992) 'Preparation and characterization of superhydrophobic FEP-Teflon surfaces', J. Adhesion Science and Technology 6, 347-356.

Campbell, C.D., Brooks, D.H., Webster, M.W., Diamond, D.L., Peel, R.L. and Bahnson, H.T. (1979) 'Expanded microporous Polytetrafluoroethylene as a vascular substitute: A two year follow-up', Surgery 85, 177-183.

Campbell, C.D., Goldfarb, D. and Detton, D.D. (1974) 'Expanded polytetrafluoroethylene as a small artery substitute', Trans. Am. Soc. Artif. Intern. Organs 20, 86-90.

Cuadros, C.L. (1984) 'One hundred procent patency of one-millimeter polytetrafluoroethylene (Gore-Tex) grafts in the carotid arteries of rats', Microsurg. 5, 1-11.

Derman, G.H. and Reichman, O.H. (1979), 'Microvascular grafting with small-diameter prostheses', Surg. Forum 30, 208-210.

Gore, R.W. (1976) Process for producing porous products, W.L. Gore and Ass. Inc. U.S. Patent 3953566, Int. Cl 264-288.

Gottlow, J., Nyman, T. and Lindhe, J. (1984) 'New attachment formation as the result of controlled tissue regeneration', J. Clin. Periodontology 11, 494-499.

Graham, L.M. and Bergan, J.J. (1982) 'Expanded polytetrafluoroethylene vascular grafts: clinical status and experimental observations', in J.C. Stanley, W.E. Burkel, S.M. Lindenauer, R.H. Bartlett and J.G. Turcotte (eds.), Biologic and Synthetic Vascular Prostheses, Grune and Stratton, NY, 563-586.

Graham, L.M., Burkel, W.E., Ford, J.W., Vinter, D.W., Kahn, R.N. and Stanley, J.C. (1980a) 'Immediate seeding of enzymatically derived endothelium on Dacron vascular grafts: early experimental studies with autogenous canine cells', Arch. Surg. 115, 1289-1294.

Graham, L.M., Vinter, D.W., Ford, J.W., Kahn, R.N., Burkel, W.E. and Stanley, J.C. (1980b) 'Endothelial seeding of prosthetic vascular grafts. early experimental studies with cultured autologous canine endothelium', Arch. Surg. 115, 929-933.

Gristina, A.G. (1987) 'Biomaterials-centered infection: microbial adhesion versus tissue integration', Science 237: 1588-1595.

Harkes, G. (1991) Interaction of *Escherichia coli* with solid surfaces - adhesion, growth and migration. Ph.D. Thesis, University of Twente, The Netherlands.

Harkes, G., Dankert, J. and Feijen, J. (1992) 'Bacterial migration along solid surfaces', Appl. Environ. Microbiol. 58, 1500-1505.

Herring, M.B., Gardner, A. and Glover, J. (1978) 'A single staged technique for seeding vascular grafts with autogenous endothelium', Surgery, 84, 498-504.

Jenkins, S.C., Klamer, T.W., Parteka, J.J. and Condon, R.E. (1983) 'A comparison of prosthetic materials used to repair abdominal wall defects', Surgery 94, 392-398.

Lamb, J.P., Vitale, T. and Kaminski, D.L. (1983) 'Comparative evaluation of synthetic meshes used for abdominal wall replacement', Surgery 93, 643-648.

Neel, H.B. (1983) 'Implants of Gore-Tex', Arch. Otolaryngol. 109, 427-433.

Nickel, J.C., Ruseska, I., Wright, J.B. and Costerton, J.W. (1985a) 'Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material', Antimicrobial agents and chemotherapy 27, 619-624.

Nickel, J.C., Grant, S.K. and Costerton, J.W. (1985b) 'Catheter-associated bacteriuria', Urology 26, 369-375.

Pilliar, R.M. (1987) 'Porous-surfaced metallic implants for orthopedic applications', J. Biomed. Mater. Res. 21, 1-33.

Sauvage, L.R., Davis, C.C., Smith, J.C., Rittenhouse, E.A., Hall, D.G., Mansfield, P.B., Schultz, G.A., Wu, H-E., Ray, L.I., Mathisen, S.R. and Usui, Y. (1987) 'Development and clinical use of porous Dacron arterial prostheses', in P.N. Sawyer (ed.), 1987 Modern Vascular Grafts, McGraw-Hill, NY, pp 225-255.

Schakenraad, J.M., Busscher, H.J., Wildevuur, Ch.R.H. and Arends, J. (1986) 'The influence of substratum surface free energy on growth and spreading of human fibroblasts in the presence and absence of serum proteins', J. Biom. Mat. Res. 20, 773-784.

Schakenraad, J.M., Stokroos, I., Bartels, H. and Busscher, H.J. (1992) 'Patency of small caliber superhydrophobic e-PTFE vascular grafts; a pilot study in the rabbit carotid artery', Cells and Materials in press.

Sedlarik, K.M., Van Wachem, P.B., Bartels, H. and Schakenraad, J.M. (1990) 'Rapid endothelialization of microporous prostheses covered with meshed vascular tissue; a preliminary report' Biomaterials 11, 4-8.

Silver, F. and Doillon, C. (1989) 'Biocompatibility - Interaction of biological and implantable materials', VCH Publishers, Inc., New York.

Soyer, T., Lempinen M. and Cooper, P. (1972) 'A new venous prosthesis', Surgery 72, 864-872.

Van Kooten, T.G., Schakenraad, J.M., Van der Mei, H.C. and Busscher, H.J. (1992a) 'Development and use of a parallel-plate flow chamber for studying cellular adhesion to solid surfaces', J. Biomed. Mater. Res. 26, 725-738.

Van Kooten, T.G., Schakenraad, J.M., Van der Mei, H.C. and Busscher, H.J. (1992b) 'Detachment of human fibroblasts from FEP-Teflon surfaces', Cells and Materials 1, 307-316.

Van der Lei, B., Robinson, P.H., Bartels, H.L. and Wildevuur, C.R.H. (1989) 'Microarterial grafting into the carotid artery of the rabbit: some considerations concerning species-dependent thrombogenicity', Br. J. Plast. Surg. 42, 59-64.

Van Oene, G.H., Yue, X., Van der Lei, B., Schakenraad, J.M., Kuit, J.H., Feijen, J. and Wildevuur, Ch. (1987) 'Smooth muscle cell seeding enhances neo-endothelialization', in Zilla, Fasol and Deutsch (eds.), Endothelialization of Vascular Grafts, Karger, Basel, 160-166.

Volder, J.G. and Kolff, W.J. (1974) 'Induced growth of connective tissue in cardiovascular prostheses', Trans. Am. Soc. Artif. Intern. Organs 20, 521-526.

Watanabe, K. (1979) 'A New Microvascular Prosthesis', Trans. VII Int. Congr. Plastic Surgery, Brazil.

Williams, D.F. (1981) 'Implants in dental and maxillofacial surgery', Biomaterials 2, 133-146

Williams, D.F. (1987) 'Definitions in Biomaterials', Elsevier, Amsterdam.

Yue, X., Van Oene, G., Schakenraad, J.M., Van der Lei, B., Kuit, J., Feyen, J. and Wildevuur, Ch.R.H. (1988) 'Smooth muscle cell seeding in biodegradable grafts in rats to enhance the process of arterial wall regeneration', Surgery, 103, 206-212.

FORMATION AND PREVENTION OF DENTAL PLAQUE - a physico-chemical approach

H.J. BUSSCHER[1], M. QUIRYNEN[2] AND H.C. VAN DER MEI[1]

[1]Laboratory for Materia Technica
University of Groningen
Antonius Deusinglaan 1
9713 AV Groningen, the Netherlands

[2]Department of Periodontology
Catholic University of Leuven
Capucynenvoer 7,
B-3000 Leuven, Belgium.

1. Features of Dental Plaque

Intra-oral surfaces inevitably become covered with bacterial deposits, named "dental plaque". Plaque consists for approximately 70% by weight out of bacteria, the remainder being proteins, exopolysaccharides and food debris (Marsh and Martin, 1984).

The presence of plaque on teeth may lead to carious lesions, particularly when mutans streptococci or lactobacillus species are present (Marsh and Martin, 1984; Bowden and Hamilton, 1989). Also periodontal diseases are unequivocally related with the presence of plaque, the causative microorganisms being here black pigmented bacteroides species (*Prevotella intermedia* and *Porphyromonas gingivalis*), *Actinobacillus actinomycetemcomitans* and spirochetes (Van der Velden *et al.*, 1986). Even in edentulous people, plaque sticking to prosthetic devices, may lead to the development of diseases. Denture stomatitis for example, is a chronic inflammation of both the gingiva and the alveolar mucosa, due to the presence of yeasts, mainly *Candida albicans*, in the plaque on dentures (Verran and Motteram, 1987; Koopmans *et al.*, 1988). Both the compositions of the supra- and subgingival plaques (see Fig. 1) vary in time. Already five minutes after perfect tooth cleaning, the first adhering bacteria, predominantly

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 327–354.

Streptococcus sanguis, and *Neisseria species*, are detectable on the tooth surface (Marsh and Martin, 1984). Within three hours a mixed bacterial flora can be found, consisting of Gram$^+$, facultative anaerobe cocci and Gram$^+$, facultative anaerobe rods. After a week of undisturbed plaque growth, the complexity of the plaque has greatly increased, including fusobacteria, filaments, spirils and spirochetes (see Fig. 2).

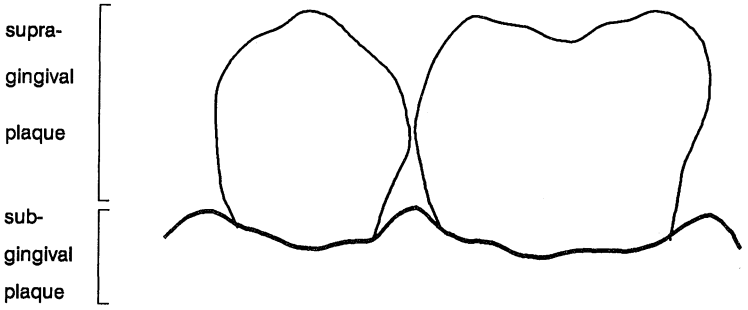


Figure 1. Illustration of the terminology used to describe supra- and subgingival plaque (adapted from Marsh and Martin, 1984).

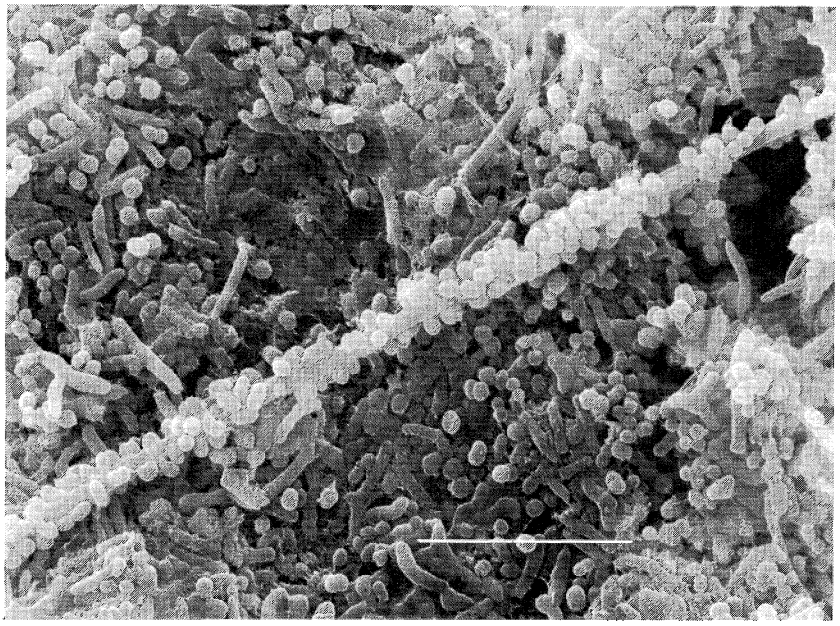


Figure 2. Scanning electron micrograph of dental plaque (courtesy of Dr. W.L. Jongebloed). Bar denotes 10 μm.

There is a surprising site-specificity (Gibbons and Van Houte, 1975; Gibbons, 1984) in the adhesion of oral microorganisms to intra-oral surfaces (see Table 1). Although this site-specificity can be approached from an ecological perspective and explained e.g. on the basis of the availability of specific nutrients, it can also be approached from a physico-chemical perspective. In a physico-chemical approach, the site-specific distribution of microbial strains through the oral cavity would be explained on the basis of the type of interaction between the cell and the surface to be colonized. It has been noticed for instance (Beighton, 1984), that bacteria isolated from discrete, intra-oral sites of monkeys differed in their affinity for hexadecane. Strains whose major habitat was the teeth, were significantly less hydrophobic than bacterial strains isolated predominantly from the mucosal surfaces, when studied after saliva-coating the bacteria.

TABLE 1. Approximate site-specific distribution (percentage of total streptococcal count) of streptococci in the oral cavity (source: Marsh and Martin, 1984).

Strains	cheek	tongue	saliva	teeth	
				supra-gingival	sub-gingival
S. mutans	-	-	1	1	13
S. sanguis	19	-	17	29	40
S. mitis	81	65	58	58	13
S. salivarius	-	31	23	-	-

2. Microbial Interactions with Solid Surfaces

A myriad of different interactions has been described in the literature by which microorganisms can adhere to a solid surface. However, these interactions are all manifestations of three basic physico-chemical forces: Lifshitz-Van der Waals forces, electrostatic forces and hydrogen bonding (Van Oss, 1991; Busscher *et al.*, 1992a). Fig. 3 shows the interaction energy between a spherical particle and a flat collector surface under various conditions according to the DLVO theory. In the DLVO theory, adhesion is thought to be mediated by attractive Lifshitz-Van der Waals forces and attractive or repulsive electrostatic forces (Rutter and Vincent, 1980):

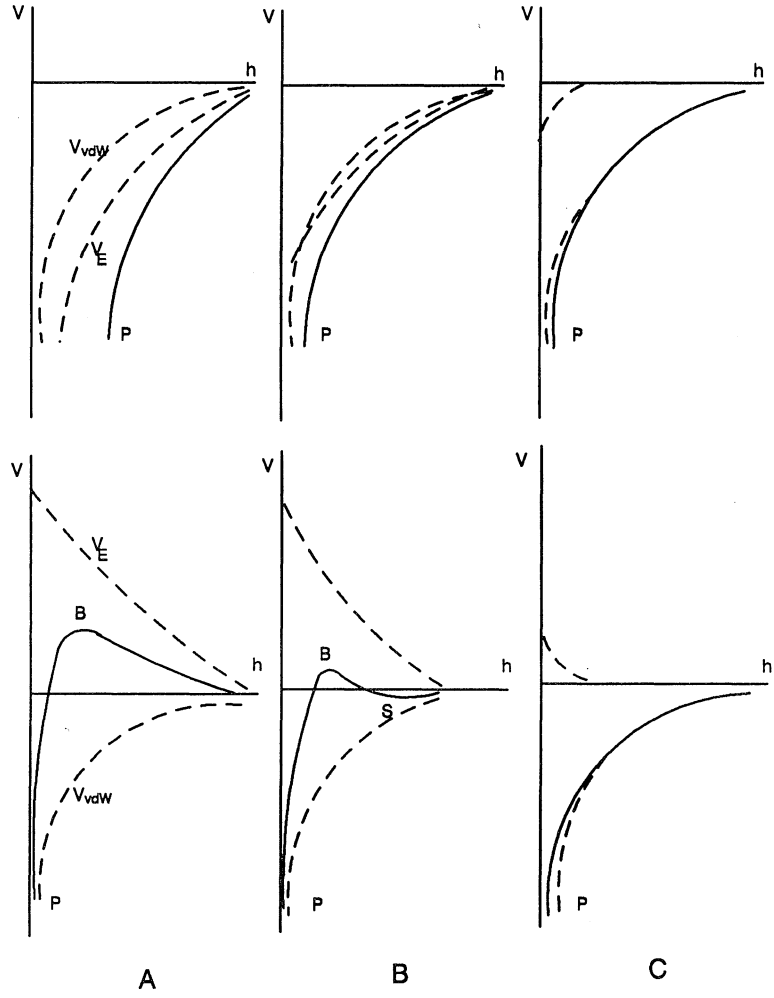


Figure 3. Interaction energies between a spherical particle and a solid substratum according to the DLVO theory as a function of distance h. Interaction energies are divided in a Lifshitz-Van der Waals part V_{vdW} and an electrostatic contribution V_E. P denotes the primary interaction minimum, B the potential energy barrier and S the secondary interaction minimum.
Top series: Oppositely charged surfaces. Bottom series: equally charged surfaces. Ionic strength of the buffers increase from A-C (adapted from Rutter and Vincent, 1980).

$$V_{total} = V_{vdW} + V_E \qquad [1]$$

$$\text{with } V_{vdW} = -\frac{A_{slb} \cdot a}{6\ h} \qquad (h \ll a) \qquad [2]$$

$$V_E = \pi\epsilon\epsilon_o.a\left(\zeta_s^2 + \zeta_b^2\right)\left(\frac{2\zeta_s\zeta_b}{\zeta_s^2 + \zeta_b^2} \cdot \ln\left(\frac{1+\exp(-\kappa h)}{1-\exp(-\kappa h)}\right) + \ln\left(1-\exp(-2\kappa h)\right)\right) \qquad [3]$$

in which A_{slb} is the Hamaker constant, a is the cell radius, h is the distance between the interacting surfaces, $\epsilon\epsilon_o$ is the di-electric constant of the medium, κ is the reciprocal Debye-Hückel length and ζ_s and ζ_b denote the zeta potentials of the solid and bacterium, respectively. Recently, Van Oss *et al.* (1986) proposed an extension of Eq. [1] to account also for hydrogen bonding.

The DLVO theory as described above is based on so-called macroscopic cell surface properties and no account is given to specific chemical and structural heterogeneities on the cell surface. Therefore, it has been argued (Busscher and Weerkamp, 1987) that the DLVO theory is only useful to describe the approach of a cell towards a substratum upto the so-called reversible, secondary minimum adhesion (see also Fig. 4). During adhesion of a cell in the secondary interaction minimum, specific groups on the cell (and possibly also on the substratum surface) can reorient themselves and strong, essentially irreversible, primary minimum adhesion occurs, often said to be mediated by specific interactions.

Although it is sometimes argued that specific interactions are due to special forces, other than those occurring in the DLVO theory, it is important to realize that also these specific interactions are caused by the same three, basic physico-chemical forces as mentioned above. However, in a specific interaction, stereo-chemical groups with a high mutual physico-chemical attraction, appear in close juxtaposition, yielding a strong bonding, as schematically depicted in Fig. 5 (Busscher *et al.*, 1992a).

In order to achieve a close juxtaposition of stereo-chemical groups, interfacial water has to be removed from in between the cell and the substratum surface. Hypothetically, removal of interfacial water may be the main mechanism (Busscher *et al.*, 1986a; Busscher and Weerkamp, 1987) by which "cell surface hydrophobicity" exerts its ubiquitously accepted (Rosenberg and Doyle, 1990) influence on bacterial adhesion. With regard to the expression "cell surface hydrophobicity" it is important to note that it is not a well-defined term in micro-

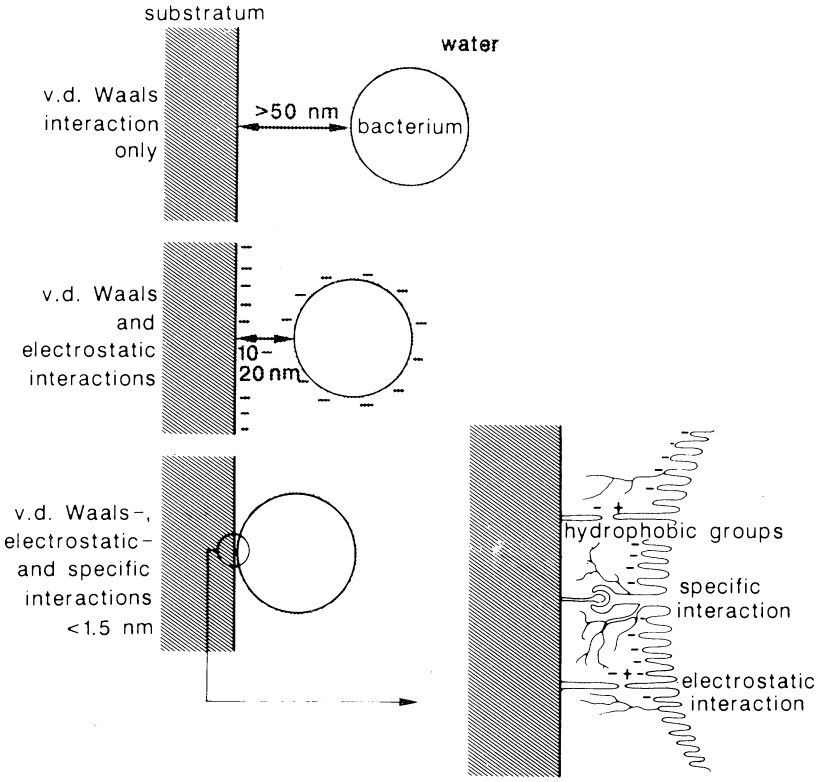


Figure 4. The importance of various types of interactions in microbial adhesion to a solid substratum, schematically depicted as a function of distance.
Three distinct interaction regions are distinguished:

- Separation distances > 50 nm, at which only Lifshitz-Van der Waals forces operate. Non-specific, macroscopic cell surface properties play the most dominating role in this stage of adhesion.
- Separation distances between 10 and 20 nm. Additional electrostatic repulsion is now becoming active, resulting in a reversible secondary minimum adhesion.
- Separation distances < 1.5 nm, only at extremely small separation distances specific interactions can take place, provided the organism is capable of extruding adhesion probes and hydrophobic groups are available to dehydrate the surfaces, facilitating direct contact. The capacity to do so will be very much strain-dependent.

(adapted from Busscher and Weerkamp, 1987)

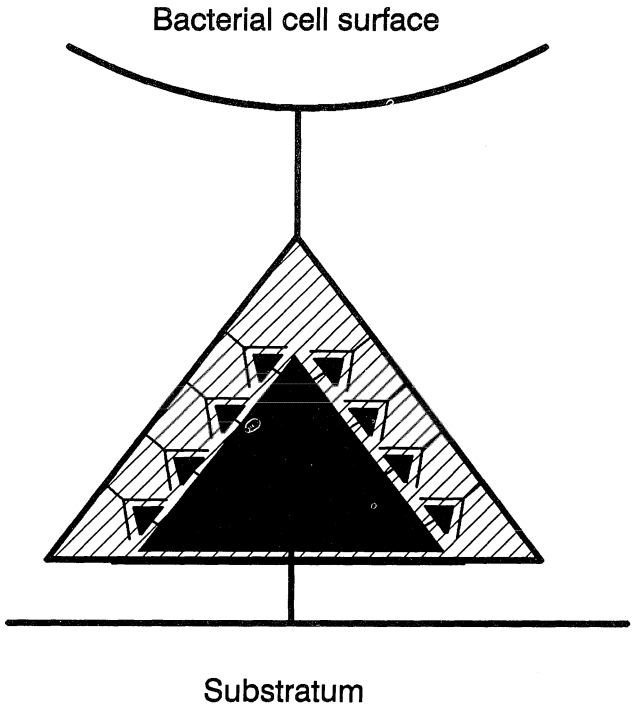


Figure 5. A specific bond, envisaged as a collection of stereo-chemical groups between which highly attractive Lifshitz-Van der Waals, electrostatic forces and hydrogen bonding exist.

biology and that there is no standard method to measure it, frequently used methods being MATH and water contact angle measurements (Van der Mei *et al.*, 1987; 1991).

Contact angle measurements also provide the basis for an other approach towards microbial adhesion than the DLVO theory, which is based on thermodynamics (Absolom *et al.*, 1983; Busscher *et al.*, 1984). In a thermodynamic approach, contact angle data are employed to derive the surface free energies γ_{sb}, γ_{sl} and γ_{bl} which are used to calculate the free energy of adhesion ΔF_{adh} according to:

$$\Delta F_{adh} = \gamma_{sb} - \gamma_{sl} - \gamma_{bl} \quad [4]$$

(For nomenclature, see Fig. 6).

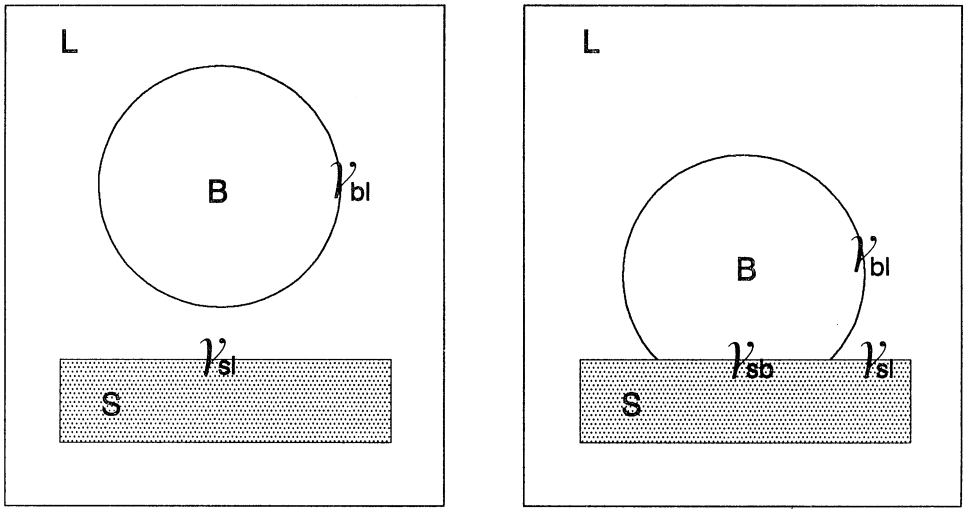


Figure 6. Interfaces involved in microbial adhesion: γ_{sl} is the solid-liquid, γ_{bl} is the bacterium-liquid-and γ_{sb} is the solid-bacterium interfacial free energy.

However, there is no agreement in physico-chemistry on how to derive surface free energies from measured contact angles (Busscher and Van Pelt, 1987; Bellon-Fontaine *et al.*, 1990) nor on the real thermodynamic status of microbial adhesion (Van Loosdrecht *et al.*, 1989).

Incidentally it is emphasized, that the thermodynamic approach is linked in some way to the DLVO theory, since the Van der Waals interaction energy in Eq. [2] is sometimes said to be equal to the Lifshitz-Van der Waals part of the free energy of adhesion in Eq. [4]

$$\Delta F_{adh}^{LW} = V_{vdW} = -\frac{A_{slb} \cdot a}{6\ h} \quad [5]$$

3. Oral Microbial Adhesion to Solid Surfaces

Hydroxyapatite is the main mineral component of dental enamel (Arends and Jongebloed, 1977). Hydroxyapatite beads, with or without a salivary coating, are therefore widely used as a model substratum, to mimic microbial adhesion to enamel (Morris and McBride, 1984; Gibbons *et al.*, 1985). However, apart from the fact that most beads used are not hydroxyapatite but tricalcium phosphate (J. Arends, private communication), hydroxyapatite beads lack the typical heterogeneity of dental enamel surfaces (see Fig. 7). More importantly, in most adhesion studies involving hydroxyapatite beads, there is no control at all of the mass transport and shear forces occurring in the system (Sjollema *et al.*, 1989a). Yet, bead studies have yielded interesting data concerning the influence of specific proteins on oral microbial adhesion.

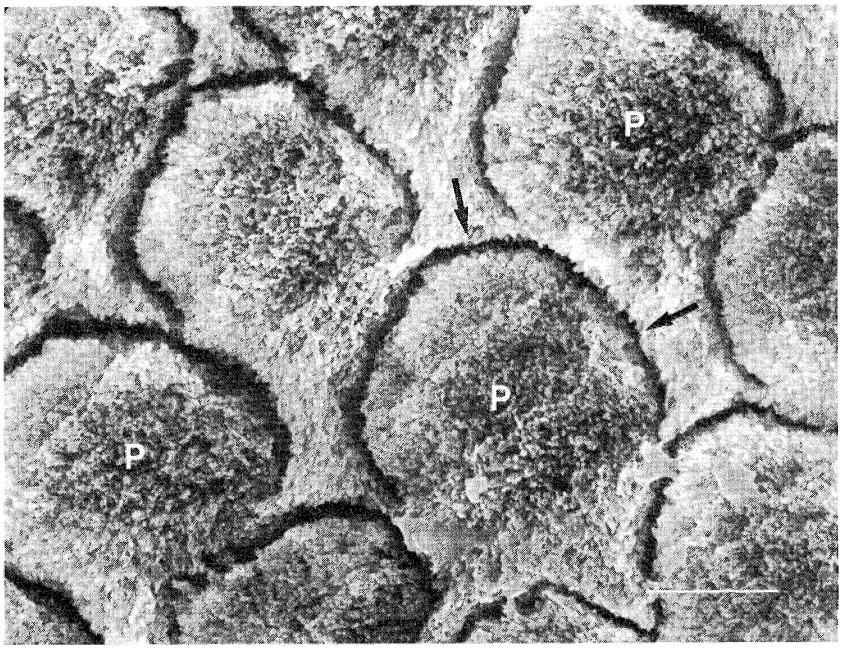


Figure 7. Scanning electron micrograph of a dental enamel surface, showing the hydroxyapatite prisms (P) and the so-called organic matrix (indicated by arrows) between the prisms (courtesy of Dr. W.L. Jongebloed). Bar denotes 3 μm.

To circumvent the mass transport problem and in order to control the shear forces, physico-chemists have developed a variety of flow cell systems to study microbial adhesion, including parallel plate (Rutter and Leech, 1980; Sjollema *et al.*, 1988) and cylindrical channels (Adamczyk and Van de Ven, 1981), rotating

disks (Mohandas *et al.*, 1974) and stagnation point flow chambers (Adamczyk *et al.*, 1985). Another major improvement of the experimental design of microbial adhesion studies involves the use of *in situ* observation techniques (Sjollema *et al.*, 1989b). The main interests of physico-chemists in (oral) microbial adhesion has been to verify the DLVO theory, the thermodynamic approach and mass transport theories. Microbiologists and biochemists, however, have focused more on the influence of salivary coatings on the adhesion. Therefore, physico-chemists have made more use of inert substrata, whereas microbiologists and biochemists have made most use of protein coated substrata.

3.1. ORAL MICROBIAL ADHESION TO INERT SUBSTRATA

Adhesion experiments to inert substrata and ground and polished enamel have shown that low surface free energy strains (e.g. *Streptococcus mitis*) adhere in higher numbers (Uyen *et al.*, 1985) and less reversibly (Busscher *et al.*, 1986b) to hydrophobic substrata than to hydrophilic substrata, while the opposite is true for high surface free energy strains. These observations are largely concurrent with thermodynamics, although Pratt-Terpstra *et al.* (1988, 1989a) proposed to introduce an additional microbial factor, that would account for the influence of surface appendages or biosurfactant production by the strains. Considering that most oral microorganisms have a high surface free energy (Van Pelt *et al.*, 1984), the above observations hold the premise that a low surface free energy coating on teeth might yield a reduced plaque formation.

A two phase adhesion process, said to indicate the transition from reversible, secondary minimum adhesion to essentially, irreversible, primary minimum adhesion (Van Loosdrecht *et al.*, 1989), has been observed for *S. sanguis* adhesion to inert substrata with different hydrophobicities (Busscher *et al.*, 1986a). Despite the fact that adhesion was generally lower on the hydrophobic substrata, the transition to the irreversible phase occurred faster, due to an easier removal of interfacial water from a hydrophobic substratum.

The initial kinetics of oral microbial adhesion to inert substrata was determined in a parallel plate flow chamber by Sjollema *et al.* (1988) and compared with the kinetics calculated on the basis of the convective-diffusion equation, while accounting for interaction forces. For most strains, the experimental deposition rates were higher than the theoretically calculated ones, which was attributed to the presence of appendages on the cell surfaces, assisting adhesion.

The use of a parallel plate flow chamber with *in situ* observation has also opened the possibility to work under conditions of varying shear, as occurring in the oral cavity. A high shear, as compared to the shear exerted by the fluid flow, on the adhering cells can be created by passing an air bubble through the chamber (Busscher *et al.*, 1990a). These experiments were originally done in our

group to demonstrate that experimental set-ups to measure microbial adhesion in which samples are passed through a liquid-air interface prior to enumeration, yield erroneous results. However, more relevantly, it appeared that less adhering bacteria were removed when an air bubble was passed through the chamber in case of adhesion from a low ionic strength buffer than in case of a high ionic strength buffer (Busscher *et al.*, 1990a). Since the electrostatic repulsion between cells and substrata is greater in case of a low ionic strength buffer, these observations may point to electrostatic attraction between positively charged domains on the cell surfaces and negatively charged substrata.

3.2. ORAL MICROBIAL ADHESION TO PROTEIN-COATED SUBSTRATA

Oral microbial adhesion to protein coated substrata has been studied traditionally to (saliva-coated) "hydroxyapatite" beads. Experiments to saliva-coated "hydroxyapatite" beads have also revealed a mechanism called "positive cooperativity" (Doyle *et al.*, 1982; Nesbitt *et al.*, 1982), which microorganisms can utilize to colonize a surface. In order to demonstrate positive cooperativity, a Scatchard analysis of the adherence data must be made, in which positive slopes indicate positive cooperativity (Doyle *et al.*, 1985), as illustrated in Fig. 8. Positive cooperativity can be characterized by stating that "adhesion of one or a few cells enhances the probability of adhesion of other cells" (Doyle, 1991). There is abundant evidence available that oral microorganisms, and particularly *S. sanguis* adhere to salivary pellicles by means of positive cooperativity (see Doyle, 1991, for an overview).

Presently, we are investigating whether the concept of "positive cooperativity" as derived from a Scatchard analysis of adhesion data is identical to the "positive cooperativity" as inferred from a nearest-neighbour analysis of the spatial arrangement of adhering cells on a substratum (Sjollema and Busscher, 1990; Sjollema *et al.*, 1990).

A comparison of adhesion data to bare substrata with those to protein-coated substrata (albumin, s-IgA, whole saliva) shows that a general effect of a protein-coating is to reduce microbial adhesion (Pratt-Terpstra *et al.*, 1987; 1989b; 1991). However, mucins isolated from human whole saliva and *Streptococcus mutans* constitute an exception in the following respect: *S. mutans* adhesion to FEP-Teflon, a very hydrophobic substratum, increased greatly after adsorption of salivary mucins, whereas hardly any effect of mucin adsorption was observed when using hydrophilic substrata (Pratt-Terpstra and Busscher, 1991). It was hypothesized that the adsorbed mucin layer was modified by the substratum hydrophobicity, revealing cryptitopes (hidden high affinity binding sites (Gibbons, 1989)) on FEP-Teflon but not on glass.

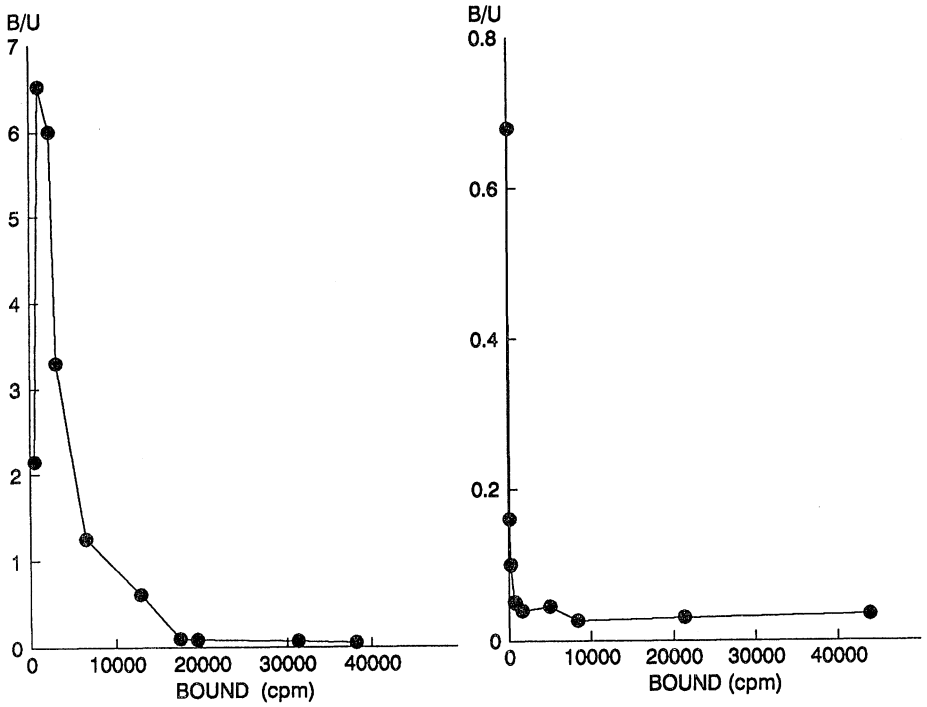


Figure 8. Scatchard representation of adhesion data: B/U versus B. According to the Scatchard analysis (Doyle *et al.*, 1985) B/U = K (N-B) in which B and U denote the number of bound and unbound cells, respectively, N reflects the number of binding sites and K is the affinity constant. Usually, B/U versus B exhibits a negative slope, however, when a positive slope occurs, this is indicative for "positive cooperativity".

Christersson *et al.* (1989) have extensively studied oral microbial retention from salivary films adsorbed on germanium prisms, as a model for the tooth surface. They found retention to be much higher on hydrophilic substrata than on hydrophobic substrata and dependent on rinse flow rates, but not affected by temperature. Thus salivary films must be different on hydrophobic than on hydrophilic substrata. Air-bubble detachment studies in a parallel plate flow chamber as described above, can in many aspects be compared with the retention studies as done by Christersson *et al.* (1989). Employing the air-bubble detachment technique, it was recently found that retention of oral

microorganisms from clean glass was strain-dependent, but that the retention capacity of different strains was equal from salivary-protein coated glass (Busscher *et al.*, 1992b). These observations lead to the conclusion that detachment of adhering bacteria from salivary films is not through interfacial failure at the film-cell interface, across which strong, irreversible specific bonds exist, but rather through cohesive failure in the salivary film or detachment of the film and cells as a whole.

4. Dental Plaque Formation on Solid Surfaces

Although numerous plaque studies have been carried out over the years, there are some that deserve special attention in view of the above mentioned premise that a low surface free energy coating on teeth might reduce plaque formation.

4.1. MODEL STUDIES ON SUPRA-GINGIVAL PLAQUE FORMATION

In vivo plaque formation can be evaluated in various ways. Glantz (1969) measured the weight of plaque formed during 7 days on materials with different surface free energies mounted on bridges in ten human volunteers. During the experimental period, the volunteers were instructed not to brush the teeth on that side of the jaw where the test piece-carrying bridge was fixed. Plaque-formation on low surface free energy materials was approximately tenfold lower than on high surface free energy materials including enamel.

Quirynen *et al.* (1988; 1989; 1990a) measured the percentage area of a tooth covered by plaque ("the planimetric plaque score"). In his studies, planimetric plaque scores were compared on polymer films with different surface free energies and/or surface roughnesses glued on the front incisors of human volunteers (see Fig. 9). These studies pointed out that even after nine days of no oral hygiene, plaque formation on hydrophobic FEP-Teflon amounted only 10-15 percent of the plaque formed on a natural tooth. However, the influence of surface free energy on plaque growth rate became less significant when the substrata were roughened. With regard to a clinical application of low surface free energy coatings, an important observation has been made by Weerkamp *et al.* (1989) that at the species level no clinically relevant differences were found in the composition of the microflora on materials with a different surface free energy, albeit that *S. sanguis* I was predominantly found on teeth, whereas *S. sanguis* II was recovered mainly from the polymeric films. Furthermore, strains isolated from FEP-Teflon were slightly more hydrophobic than those isolated from less hydrophobic substrata.

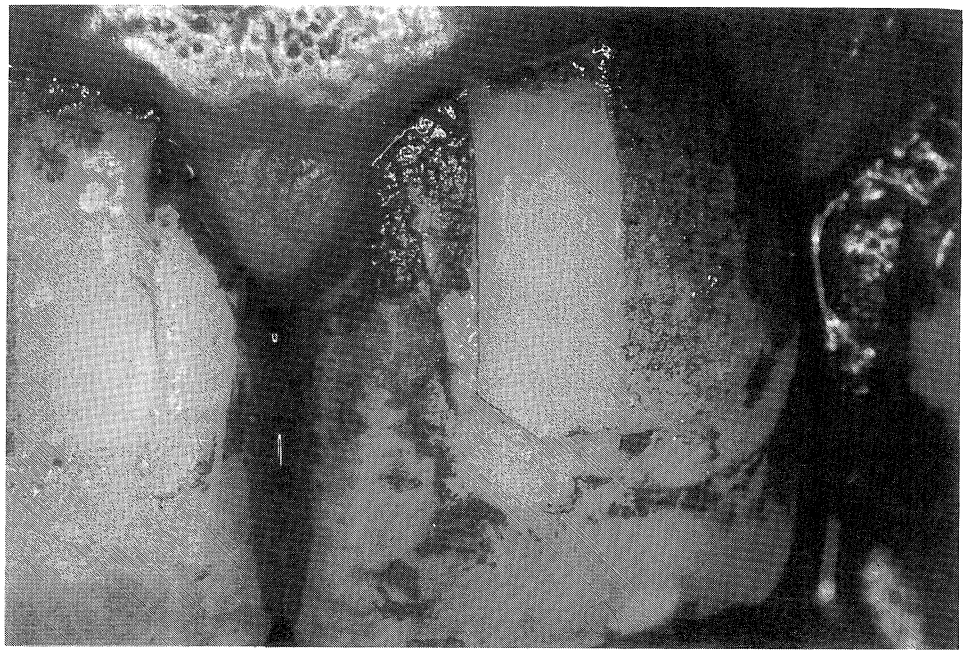


Figure 9. A comparison of the amount of plaque formed *in vivo* during 6 days on a FEP-Teflon strip, half of which was roughened (right side), while the remaining part was smooth. Note that considerably more plaque is present on the natural tooth surface. For details see Quirynen *et al.* (1988, 1989).

A third relevant study in this respect has recently been done (Rykke and Rölla, 1990; Rölla *et al.*, 1991) demonstrating that the teeth pretreated with silicone oil attracted less plaque on a 0, 1, 2, 3 scale ("the Silness-Loë plaque index (Loë, 1967)) than natural teeth. In addition, amino-acid analyses of the salivary films on silicone-oil treated and natural teeth, indicated that different films had adsorbed.

4.2. MODEL STUDIES ON SUB-GINGIVAL PLAQUE FORMATION

The above *in vivo* studies indicate a strong correlation between supra-gingival plaque formation on smooth surfaces and substratum surface free energy. Sub-gingival plaque formation not only involves different species and adhesion to both hard and soft tissues, but also occurs under less dynamic conditions (i.e. more constant shear forces) than in the supra-gingival area. The less dynamic sub-gingival conditions might be the reason why no significant differences have been found (Quirynen *et al.*, 1991) in *in vivo* sub-gingival plaque formation on

hydrophobized abutments on osseointegrated Brånemark implants as compared to the original titaniumoxide ones (see Fig. 10 for details concerning the experimental set-up).

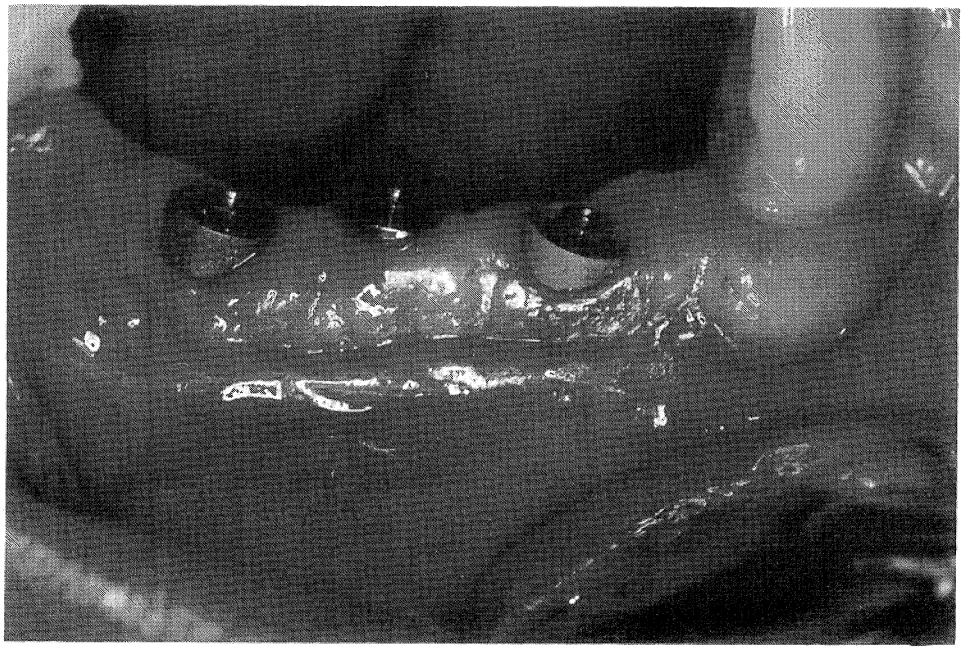


Figure 10. Experimental set-up to assess sub-gingival plaque on the abutments of osseointegrated Brånemark implants. The three abutments which normally support a partial bridge have been replaced by:
a. a Teflon-coated abutment (right);
b. a new, authentic titaniumoxide abutment (middle);
c. a roughened titaniumoxide abutment (left).
For details see Quirynen *et al.* (1991).

5. Prevention of Dental Plaque Formation

5.1. GENERAL PREVENTIVE MEASURES

Generally used preventive measures traditionally involve toothbrushing, the use of tooth picks and dental floss. As an addendum to these measures, mouthrinses become more and more in vogue, although it must be doubted whether they have a significant clinical effect, apart from creating the experience of a fresh breath. Chlorhexidine based products Peridex® and Hibident® constitute an

exception to the above (Addy, 1986) and have been shown effective in both 0.12% and 0.2% concentrations. Due to unfavourable side-effects (discolouration of teeth and mucosa, loss of taste) long-term use is not advisable (Flötra *et al.*, 1971). For mentally retarded, disabled or people after oral surgery, mouthrinses are a major means to perform oral hygiene (Hull, 1980).

Fig. 11 diagrammatically summarizes the various modes of action of a mouthrinse. Adsorption to intra-oral surfaces is not only important as a potential way to decrease the tooth surface free energy, but also since adsorbed anti-microbials may slowly desorb, thus establishing an inhibitory dose in the oral cavity during longer times than the use of the rinse. This is most often required for a sufficient kill of the microorganisms (Goodson, 1989; Scheie, 1989).

Because mouthrinses are not only an excellent vehicle to administer anti-microbials, but possibly also hydrophobizing agents, industrial interests in the development of new products remain large.

5.2. MOUTHRINSES TO PREVENT DENTAL PLAQUE FORMATION

The number of commercially available mouthrinses is huge and their supra-gingival effectiveness is usually easily proven in a short- or long term study with respect to a suitably selected placebo. Routine use, without irrigation techniques, does not allow a thorough penetration of a mouthrinse in pockets. Hence the sub-gingival effectiveness of mouthrinses is generally low.

Perdok *et al.* (1989; 1990a; 1990b; 1991) have extensively evaluated several aspects of six commercial products in one short-term clinical study, therewith providing a basis for a rigorous comparison of their clinical merits. Results on their collection of products will be used as a guideline in the forthcoming sections on anti-microbial and physico-chemical aspects of mouthrinses.

5.3. ANTIMICROBIAL ASPECTS OF COMMERCIALLY AVAILABLE MOUTHRINSES

The anti-microbial potency of an active component of a mouthrinse is usually expressed as a Minimal Inhibitory Concentration (MIC). At present, chlorhexidine (MIC: 2-32 μg/mL for $Gram^+$ and 0.5-4 μg/mL for $Gram^-$ oral bacteria) and cetylpyridinium chloride (MIC: 10^3-10^4 μg/mL for $Gram^+$ oral bacteria) are amongst the most used anti-microbials in dentistry (Goodson, 1989). However, a complete product contains many more ingredients that can influence the potency of the active component (Pader, 1985; Quirynen *et al.*, 1990b). Therefore, Perdok *et al.* (1989) defined a so-called Maximum Inhibitory Dilution (MID) indicating the maximum dilution of a complete product, still showing *in vitro* growth reduction of cells. Table 2 summarizes MID's for several products with different active components.

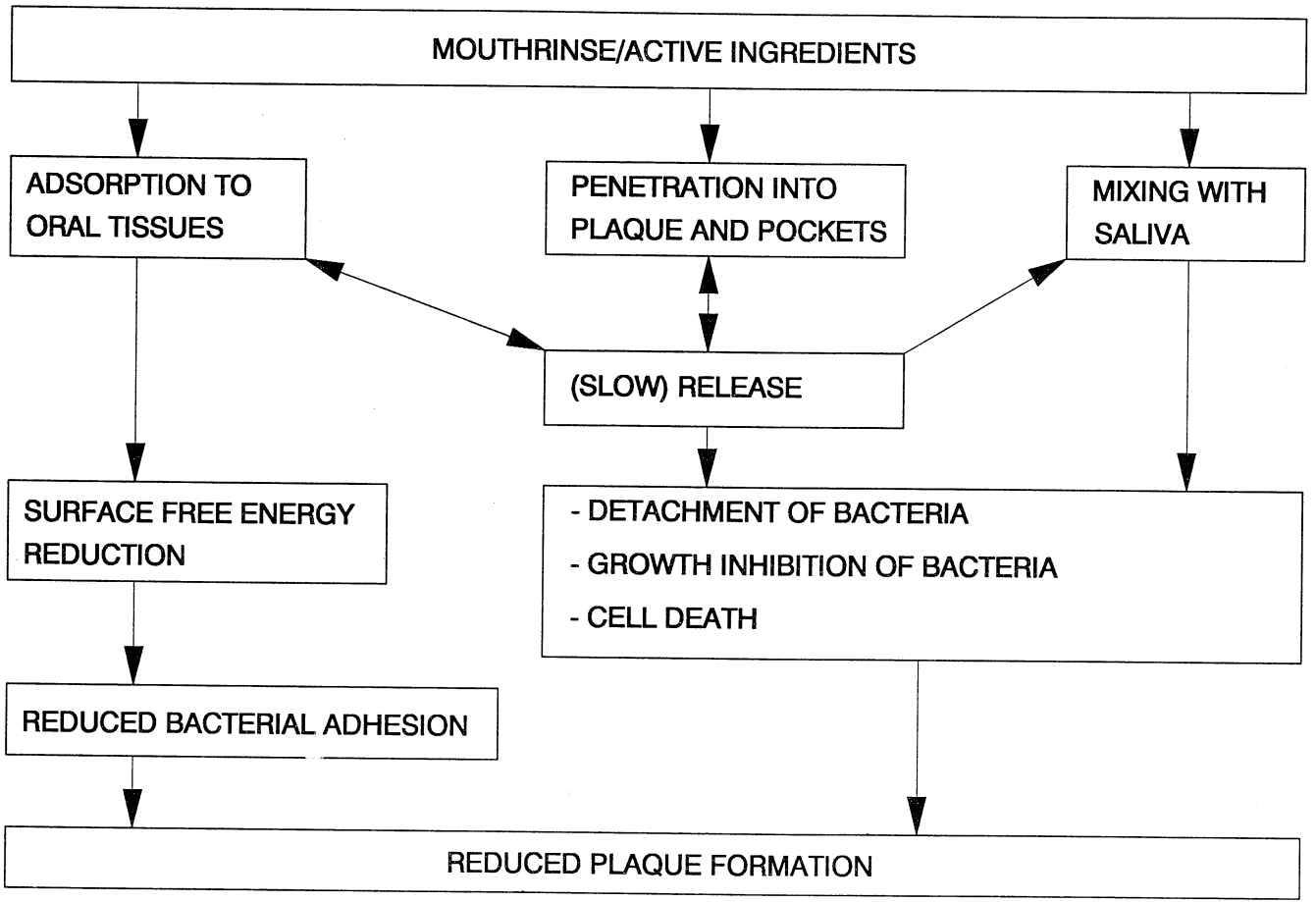


Figure 11. Diagrammatic summary of the various modes of action of a mouthrinse.

Although such data are difficult to extrapolate to the *in vivo* situation due to differences in oral dilution and contact time, Table 2 shows that the clinically most efficient products indeed can be diluted most without loosing their growth inhibiting effect.

TABLE 2. Maximum Inhibitory Dilutions (MID) of commercially available mouthrinses with respect to various oral microbial strains (data taken from Perdok *et al.*, 1989).

Product	active component	*S. mutans*	*S. sanguis*	*L. acidophilus*
Hibident®	chlorhexidine	1750 x	1750 x	2750 x
Prodent®	sodium fluoride	6 x	14 x	12 x
Meridol®	aminefluoride/stannous fluoride	1500 x	2000 x	1750 x
Merocet®	cetylpyridiniumchloride	1000 x	875 x	625 x
Listerine®	phenolic compounds	12 x	36 x	90 x
Veadent®	sanguinarine	60 x	70 x	50 x

5.4. PHYSICO-CHEMICAL ASPECTS OF COMMERCIALLY AVAILABLE MOUTHRINSES

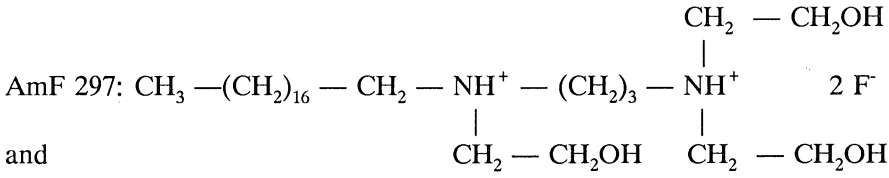
Both *in vitro* as well as *in vivo* experiments hold the premise that a low surface free energy coating on teeth would yield less plaque formation. Despite the fact that this premise exists at least since the 1960's, no commercial products with a dental application have been developed here upon. Perdok *et al.* (1991) evaluated the effect of six commercial products on the tooth wettability and concluded that none of them had any significant effect (see also Table 3). Both aminefluorides (De Jong *et al.*, 1984) as well as stannous fluoride (Glantz, 1969) have been shown to have a surface free energy reducing effect when applied on ground and polished enamel (see also the next section), hence it is particularly surprising that Meridol® does not have any effect on the tooth wettability.

In the next sections, different types of surfactants with (potential) dental applications will be described, which might exert a beneficial *in vivo* effect on plaque formation based on physico-chemical mechanisms.

TABLE 3. Clinically registered water contact angles on teeth *in vivo*:
day 0 : entry
day 14 : after two weeks use of a standard tooth paste
day 20 : after 6 days use of a given mouthrinse
± denotes the S.D. over 10 participants (data taken from Perdok *et al.*, 1991).

product	water contact angle in degrees at day		
	0	14	20
Hibident®	44±20	62±11	57±16
Prodent®	50+11	60±11	55±10
Meridol®	62±13	47±17	51± 9
Merocet®	61±13	66±11	61±12
Listerine®	55±12	55±14	59±12
Veadent®	61±11	68±15	51±10

5.4.1. *Aminefluorides*. Two types of aminefluorides are currently on the market in the Western-European continent,



AmF 297: $CH_3-(CH_2)_{16}-CH_2-NH^+(CH_2-CH_2OH)-(CH_2)_3-NH^+(CH_2-CH_2OH)(CH_2-CH_2OH)$ $2\,F^-$

and

AmF 335: $CH_3-(CH_2)_7-CH=CH-(CH_2)_7-CH_2-NH_3^+$ F^-

Applied in a tooth paste, these surfactants leave CaF_2-like globular deposits on the tooth surface which might enhance enamel remineralization (see Fig. 12a). However, aminefluorides also have a pronounced anti-microbial potency (Perdok *et al.*, 1988; 1989) and adsorb very well to ground and polished enamel (De Jong *et al.*, 1984; Busscher *et al.*, 1988). Both surfactants decrease the enamel surface free energy when adsorbed, while AmF has the tendency to form double layers when applied for a long time or in high concentrations (De Jong *et al.*, 1984). Adsorbed to salivary films, however, aminefluorides appear not to have a surface free energy reducing effect (see Fig. 12b,c), possibly due to the fact that their adsorption to salivary proteins is different than their adsorption to ground and polished enamel (Busscher *et al.*, 1988; 1990a).

Figure 12. Overview of different effects of aminefluoride adsorption on enamel surfaces.

a: Scanning electron micrograph of enamel after application of aminefluorides. Arrows indicate calciumfluoride deposits (courtesy of Dr. W.L. Jongebloed). Bar is 1 μm.

5.4.2. *Perfluoralkyl Surfactants*. These non-bacteriocidal surfactants have been described by Gaffar *et al.* (1987) for dental applications. Among the three anionic perfluoralkyl surfactants tested, only perfluorosulfonamidoalkyl esters of phosphorous acid (PSAEP) were reported to yield a 66% inhibition of bacterial adhesion to saliva-coated hydroxyapatite disks treated with this surfactant. Also the occurrence of *S. mutans* induced dental caries in rats was found to decrease significantly after topical application of the surfactant. Although it was suggested that PSAEP works by adsorption of its charged phosphate groups towards the surface, while its perfluoralkane chain renders a hydrophobic surface, this suggestion was unfortunately not verified by contact angle measurements. Obviously, a mouthrinse on the basis of PSAEP is less likely to adversely affect the normal microbial ecology in the oral cavity.

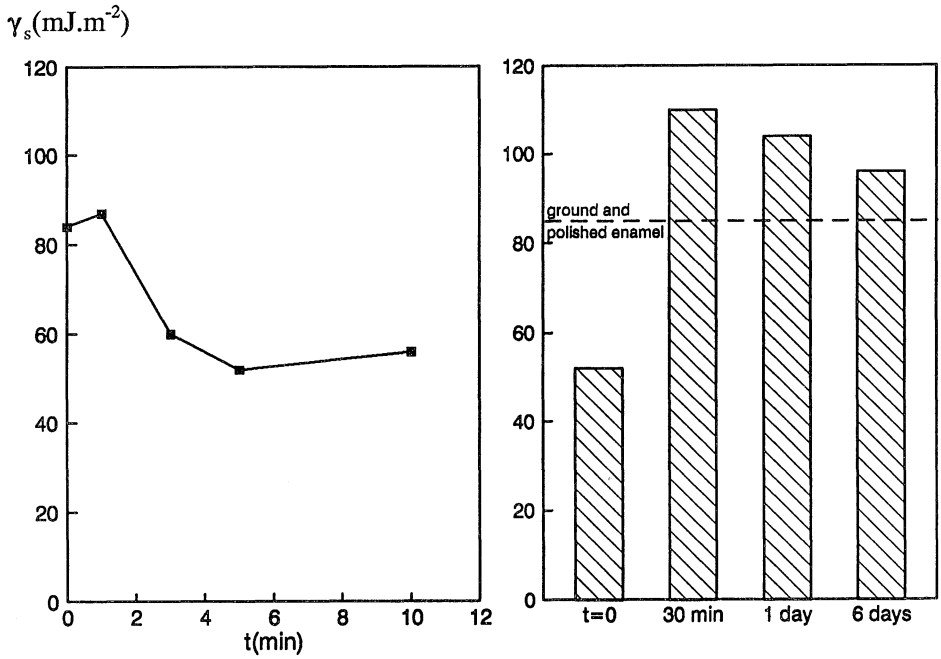
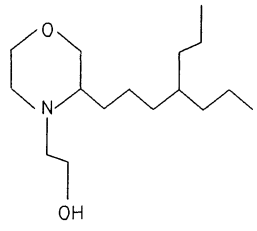


Figure 12. Overview of different effects of aminefluoride adsorption on enamel surfaces.

b (left): Surface free energies γ_s of ground and polished enamel after application of an aminefluoride as a function of the application time t. Adapted from De Jong *et al.* (1984).

c (right): *In vivo* effects of pellicle formation on the surface free energy of aminefluoride treated enamel. At t=0, ground and polished enamel samples treated with aminefluorides were put in the flanks of dentures of human volunteers and worn for the indicated times. Adapted from Busscher *et al.* (1988).

5.4.3. *Amino Alcohols.* Amino alcohols are presently being used in a new product, Delmopinol,



and the claims are that its clinical efficacy is similar to the ones of chlorhexidine based products but a 5-125 fold lower anti-microbial potency is reported too (Simonsson *et al.*, 1991a). Hence its mode of action is likely to be based on physico-chemical mechanisms.

Delmopinol is a low molecular weight surfactant and its working mechanism is hypothesized to be based on penetration into the plaque, therewith disrupting its cohesion. Also Delmopinol stimulated desorption of salivary pellicles from hydrophobic surfaces, but not from hydrophilic ones. Furthermore treatment of oral bacteria with Delmopinol reduced the hydrophobicity of the cell surfaces, possibly also by removal of surface proteins (Simonsson *et al.*, 1991b). This might provide a basis for the physico-chemical mode of action of the product.

6. Acknowledgements

The authors are greatly indebted to Mrs. E. van Drooge and Mrs. M.P. Schakenraad-Dolfing for the preparation of this manuscript.

7. References

Absolom, D.R., Lamberti, F.V., Policova, Z., Zingg, W., Van Oss, C.J. and Neumann, A.W. (1983) 'Surface thermodynamics of bacterial adhesion', Appl. Environ. Microbiol. 46, 90-97.

Adamczyk, Z. and Van de Ven, T.G.M. (1981) 'Deposition of particles under external forces in laminar flow through parallel plate and cylindrical channels', J. Coll. Interface Sci. 80, 340-356.

Adamczyk, Z., Zembala, M. Siwek, B. and Czarnecki, J. (1985) 'Kinetics of latex particle deposition from flowing suspensions', J. Coll. Interface Sci. 110, 188-200.

Addy, M. (1986) 'Chlorhexidine compared with other locally delivered antimicrobials - A short review', J. Clin. Periodontol. 13, 957-964.

Arends J. and Jongebloed, W.L. (1977) 'The enamel substrate-characteristics of the enamel surface', Swed. Dent. J. 1, 215-224.

Beighton, D. (1984) 'The influence of saliva on the hydrophobic surface properties of bacteria isolated from oral sites of macaque monkeys', FEMS Microbiol. Lett. 21, 239-242.

Bellon-Fontaine, M.N., Mozes, N., Van der Mei, H.C., Sjollema, J., Cerf, O., Rouxhet, P.G. and Busscher, H.J. (1990) 'A comparison of thermodynamic approaches to predict the adhesion of dairy microorganisms to solid substrata', Cell Biophysics 17, 93-106.

Bowden, G.H.W. and Hamilton, I.R. (1989) 'Competition between *Streptococcus mutans* and *Lactobacillus casei* in mixed continuous culture', Oral Microbiol. Immunol. 4, 57-64.

Busscher, H.J., Weerkamp, A.H., Van der Mei, H.C., Van Pelt, A.W.J., De Jong, H.P., and Arends J. (1984) 'Measurements of the surface free energy of bacterial cell surfaces and its relevance for adhesion', Appl. Environ. Microbiol. 48, 980-983.

Busscher, H.J., Uyen, M.H.W.J.C., Van Pelt, A.W.J., Weerkamp, A.H. and Arends, J. (1986a) 'Kinetics of adhesion of the oral bacterium *S. sanguis* CH3 to polymers with different surface free energies', Appl. Environ. Microbiol. 51, 910-914.

Busscher, H.J., Uyen, M.H.M.J.C., Weerkamp, A.H., Postma, W.J. and Arends, J. (1986b) 'Reversibility of adhesion of oral streptococci to solids', FEMS Microbiol. Lett. 35, 303-306.

Busscher, H.J. and Weerkamp, A.H. (1987) 'Specific and non-specific interactions in bacterial adhesion to solid substrata', FEMS Microbiol. Rev. 46, 165-173.

Busscher, H.J. and Van Pelt, A.W.J. (1987) 'The possibility of deriving solid surface free energies from contact angles measurements with one liquid'. J. Mat. Sci. Lett. 6, 815-816.

Busscher, H.J., Uyen, H.M., De Jong, H.P., Kip, G.A.M. and Arends, J. (1988) 'Adsorption of aminefluorides on human enamel', J. Dent. 16, 166-171.

Busscher, H.J., Sjollema, J. and Van der Mei, H.C. (1990a) 'Relative importance of surface free energy as a measure of hydrophobicity in bacterial adhesion to solids', in R.J. Doyle and M. Rosenberg (eds.), Microbial Cell Surface Hydrophobicity, American Society for Microbiology, Washington, pp. 335-359.

Busscher, H.J., Van der Mei, H.C., Genet, M.J., Perdok, J.F. and Rouxhet, P.G. (1990b) 'XPS determination of the thickness of adsorbed mouthrinse components on dental enamel', Surf. Interf. Anal. 15, 344-346.

Busscher, H.J., Cowan, M.M., and Van der Mei, H.C. (1992a) 'On the relative importance of specific and non-specific approaches to oral microbial adhesion', FEMS Microbiol. Rev. in press.

Busscher, H.J., Doornbusch, G.I. and Van der Mei, H.C. (1992b) 'Adhesion of mutans streptococci to glass with and without a salivary coating as studied in a parallel plate flow chamber', J. Dent. Res. 71, 491-500.

Christersson, C.E., Dunford, R.G. and Glantz, P.O. (1989) 'Effect of critical surface tension on retention of oral microorganims', Scan. J. Dent. Res. 97, 247-256.

De Jong, H.P., Van Pelt, A.W.J., De Boer, P., Busscher, H.J. and Arends, J. (1984) 'The effect of topical fluoride applications on the surface free energy of human enamel -an *in vitro* study-', J. Dent. Res. 63, 635-641.

Doyle, R.J., Nesbitt, W.E. and Taylor, K.G. (1982) 'On the mechanism of adherence of *Streptococcus sanguis* to hydroxylapatite', FEMS Microbiol. Lett. 15, 1-5.

Doyle, R.J., Oakley, D., Murphy, K., McAlister, D. and Taylor, K.G. (1985) 'Graphical analyses of microbial adherence data' in S. Mergenhagen and B. Rosan (eds.), Molecular Basis of Oral Microbial Adhesion, American Society for Microbiology, Washington D.C., pp. 109-113.

Doyle, R.J. (1991) 'Strategies in experimental microbial adhesion research' in N. Mozes, P.S. Handley, H.J. Busscher and P.G. Rouxhet (eds.), Microbial Cell Surface Analysis - Structural and Physico-Chemical Methods, VCH Publishers, Inc., New York, pp. 291-316.

Flötra, L., Gjermo, P. and Waerhaug, J. (1971) 'Side effects of chlorhexidine mouthwashes' Scan. J. Dent. Res. 79, 119-125.

Gaffar, A., Esposito, A., Bahl, M. Steinberg, L. and Mandel, I. (1987) 'Interaction of perfluoralkyl surfactants to teeth *in vitro* and *in vivo*', Colloids Surf. 26, 109-121.

Gibbons, R.J. and Van Houte, J. (1975) 'Bacterial adherence in oral microbial ecology', Annu. Rev. Microbiol. 29, 19-44.

Gibbons, R.J. (1984) 'Microbial ecology, adherent interactions which may affect microbial ecology in the mouth', J. Dent. Res. 63, 378-385.

Gibbons, R.J., Etherden, I. and Morenco, E.C. (1985) 'Contribution of stereochemical interactions in the adhesion of *Streptococcus sanguis* C5 to experimental pellicles', J. Dent. Res. 64, 96-101.

Gibbons, R.J. (1989) 'Bacterial adhesion to oral tissues: a model for infectious diseases', J. Dent. Res. 68, 750-760.

Glantz, P.O. (1969) 'On wettability and adhesiveness', Odont. Revy. 20 (suppl. 17), 5-124.

Goodson, J.M. (1989) 'Pharmacokinetic principles controlling efficacy of oral therapy', J. Dent. Res. 68, 1625-1632.

Hull, P.S. (1980) 'Chemical inhibition of plaque', J. Clin. Periodontol. 7, 431-442.

Koopmans, A.S.F., Kippuw, N. and De Graaff, J. (1988) 'Bacterial involvement in denture-induced stomatitis', J. Dent. Res. 67, 1246-1250.

Loë, N. (1967) 'The gingival index, the plaque index and retention index systems', J. Periodontol. 28, 610-616.

Marsh, P. and Martin, M. (1984) 'Oral Microbiology', American Society for Microbiology, Washington.

Mohandas, N., Hochmuth, R.M. and Spaeth, E. (1974) 'Adhesion of red cells to foreign surfaces in the presence of flow', J. Biomed. Mat. Res. 8, 119-136.

Morris, E.J. and McBride, B.C. (1984) 'Adherence of *Streptococcus sanguis* to saliva-coated hydroxyapatite: evidence for two binding sites', Infect. Immun. 43, 656-663.

Nesbitt, W.E., Doyle, R.J., Taylor, K.G., Staat, R.H. and Arnold, R.R. (1982) 'Positive cooperativity in the binding of *Streptococcus sanguis* to hydroxyapatite', Infect. Immun. 35, 157-165.

Pader, M. (1985) 'Oral hygiene products', in M.M. Rieger (ed.), Surfactants in Cosmetics, Marcel Dekker Inc., New York, pp. 293-348.

Perdok, J.F., Busscher, H.J., Weerkamp, A.H. and Arends, J. (1988) 'The effect of an aminefluoride-stannousfluoride containing mouthrinse on enamel surface free energy and the development of plaque and gingivitis', Clin. Prev. Dent. 10, 3-9.

Perdok, J.F., Van der Mei, H.C. and Busscher, H.J. (1989) 'A comparison of bacterial growth inhibiting effects of six commercially available mouthrinses', Microbial Ecology in Health and Disease 2, 191-196.

Perdok, J.F., Van der Mei, H.C. and Busscher, H.J. (1990a) 'Physico-chemical properties of commercially available mouthrinses', J. Dent. 18, 147-150.

Perdok, J.F., Van der Mei, H.C., Busscher, H.J., Genet, M.J. and Rouxhet, P.G. (1990b) 'Surface free energies and elemental surface compositions of human enamel after application of commercially available mouthrinses and adsorption of salivary constituents', J. Clin. Dent. 2, 43-47.

Perdok, J.F., Van der Mei, H.C. and Busscher, H.J. (1991) 'Clinical effects of commercially available mouthrinses on the development of plaque, gingivitis and enamel surface free energy', Biofouling 3, 209-221.

Pratt-Terpstra, I.H., Weerkamp, A.H. and Busscher, H.J. (1987) 'Adhesion of oral streptococci from a flowing suspension to uncoated and albumin-coated surfaces', J. Gen. Microbiol. 133, 3199-3206.

Pratt-Terpstra, I.H., Weerkamp, A.H. and Busscher, H.J. (1988) 'On a relation between interfacial free energy-dependent and non-interfacial free energy-dependent adherence of oral streptococci to solid substrata', Curr. Microbiol. 16, 311-313.

Pratt-Terpstra, I.H., Weerkamp, A.H. and Busscher, H.J. (1989a) 'Microbial factors in a thermodynamic approach of oral streptococcal adhesion to solid substrata', J. Coll. Interface Sci. 129, 568-574.

Pratt-Terpstra, I.H., Weerkamp, A.H. and Busscher, H.J. (1989b) 'The effects of pellicle formation on streptococcal adhesion to enamel and artificial substrata with various surface free energies', J. Dent. Res. 68, 463-467.

Pratt-Terpstra, I.H. and Busscher, H.J. (1991) 'Adsorption of salivary mucins onto enamel and artificial solid substrata and its influence on oral streptococcal adhesion', Biofouling 3, 199-207.

Pratt-Terpstra, I.H., Mulder, J., Weerkamp, A.H., Feijen, J. and Busscher, H.J. (1991) 'Secretory IgA adsorption and oral streptococcal adhesion to human enamel and artificial substrata with various surface free energies', J. Biomat. Sci. Polymer edn. 2, 239-253.

Quirynen, M., Marechal, M., Busscher, H.J., El-Abiad, M., Arends, J. and Van Steenberghe, D. (1988) 'The influence of surface characteristics on the early bacterial colonization of intra-oral surfaces', J. Clin. Dent. 1, 14-19.

Quirynen, M. Marechal, M., Busscher, H.J., Weerkamp, A.H., Arends, J. and Van Steenberghe, D. (1989) 'The influence of surface free energy on planimetric plaque growth in man', J. Dent. Res. 68, 796-799.

Quirynen, M., Marechal, M., Busscher, H.J., Weerkamp, A.H., Darius, P.L. and Van Steenberghe, D. (1990a) 'The influence of surface free energy and surface roughness on early plaque formation - An *in vivo* study in man', J. Clin. Periodontol. 17, 138-144.

Quirynen, M., Marechal, M. and Van Steenberghe, D. (1990b) 'Comparative antiplaque activity of sanguinarine and chlorhexidine in man', J. Clin. Periodontol. 17, 223-227.

Quirynen, M., Marechal, M., Van Steenberghe, D., Busscher, H.J. and Van der Mei, H.C. (1991) 'The bacterial colonization of intra-oral hard surfaces *in vivo*: influence of surface free energy and surface roughness', Biofouling 4, 187-198.

Rölla, G., Ellingson, J.E. and Halofson, B. (1991) 'Enhancement and inhibition of dental plaque formation - some old and new concepts', Biofouling 3, 175-181.

Rosenberg, M. and Doyle, R.J. (1990) 'Microbial cell surface hydrophobicity: History, measurement and significance', in R.J. Doyle and M. Rosenberg (eds.), Microbial Cell Surface Hydrophobicity, American Society for Microbiology, Washington, pp. 1-38.

Rutter, P.R. and Leech, R. (1980) 'Deposition of *Streptococcus sanguis* NCTC 7868 from a flowing suspension', J. Gen. Microbiol. 120, 301-307.

Rutter, P.R. and Vincent, B. (1980) 'The adhesion of microorganisms to sufaces: physico-chemical aspects', in R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter and B. Vincent (eds.), Microbial Adhesion to Surfaces, Ellis Horwood, Chichester, pp. 79-93.

Rykke, M. and Rölla, G. (1990) 'Effect of silicone oil on protein adsorption to hydroxyapatite *in vitro* and on pellicle formation *in vivo*', Scan. J. Dent. Res. 98, 401-411.

Scheie, A.A. (1989) 'Modes of action of currently known chemical anti-plaque agents other than chlorhexidine', J. Dent. Res. 68, 1609-1616.

Simonsson, T., Hoid, E.B., Rundegren, J. and Edwardsson, S. (1991a) 'Effect of Delmopinol on *in vitro* dental plaque formation, bacterial acid production and the number of microorganisms in human saliva', Oral Microbiol. Immunol. 6, 305-309.

Simonsson, T., Arnebrant, T. and Petersson, L. (1991b) 'The effect of Delmopinol on salivary pellicles, the wettability of tooth surfaces *in vivo* and bacterial cell surfaces *in vitro*', Biofouling 3, 251-260.

Sjollema, J., Busscher, H.J. and Weerkamp, A.H. (1988) 'Deposition of oral streptococci and polystyrene particles onto glass in a parallel plate flow cell', Biofouling 1, 101-112.

Sjollema, J., Busscher, H.J. and Weerkamp, A.H. (1989a) 'Experimental approaches for studying adhesion of microorganisms to solid substrata: applications and mass transport', J. Microbiol. Meth. 9, 79-90.

Sjollema, J., Busscher, H.J. and Weerkamp, A.H. (1989b) 'Real-time image analysis of adhering microorganisms in a parallel plate flow cell using automated image analysis', J. Microbiol. Meth. 9, 73-78.

Sjollema, J. and Busscher, H.J. (1990) 'Deposition of polystyrene particles in a parallel plate flow cell. 2- Pair distribution functions between deposited particles', Colloids Surf. 47, 337-352.

Sjollema, J., Van der Mei, H.C., Uyen, H.M. and Busscher, H.J. (1990) 'Direct observation of cooperative effects in oral streptococcal adhesion to glass by analysis of the spatial arrangement of adhering bacteria', FEMS Microbiol. Lett. 69, 263-270.

Uyen, H.M., Busscher, H.J., Weerkamp, A.H. and Arends, J. (1985) 'Surface free energies of oral streptococci and their adhesion to solids', FEMS Microbiol. Lett. 30, 103-106.

Van der Mei, H.C., Weerkamp, A.H. and Busscher, H.J. (1987) 'A comparison of methods to determine hydrophobic properties of streptococcal cell surfaces', J. Microbiol. Meth. 6, 277-287.

Van der Mei, H.C., Rosenberg, M. and Busscher, H.J. (1991) 'Assessment of microbial cell surface hydrophobicity', in N. Mozes, P.S. Handley, H.J. Busscher and P.G. Rouxhet (eds.), Microbial Cell Surface Analysis - Structural and Physico-chemical Methods, VCH Publishers, New York, pp. 263-287.

Van der Velden, U., Van Winkelhoff, A.J., Abbas, F. and De Graaff, J. (1986) 'The habitat of periodontopathic micro-organisms', J. Clin. Periodontol. 13, 243-248.

Van Loosdrecht, M.C.M., Lyklema, J., Norde, W. and Zehnder, A.J.B. (1989) 'Bacterial adhesion: A physico-chemical approach', Microbial Ecology 17, 1-15.

Van Oss, C.J., Good, R.J. and Chaudhury, M. (1986) 'The role of Van der Waals forces and hydrogen bonds in hydrophobic interactions between biopolymers and low energy surfaces', J. Coll. Interf. Sci. 111, 378-390.

Van Oss, C.J. (1991) 'Interaction forces between biological and other polar entities in water: How many different primary forces are there?', J. Disp. Sci. Technol. 12, 201-219.

Van Pelt, A.W.J., Van der Mei, H.C., Busscher, H.J., Arends, J. and Weerkamp, A.H. (1984) 'Surface free energies of oral streptococci', FEMS Microbiol. Lett. 25, 279-282.

Verran, J. and Motteram, K.L. (1987) 'The effect of adherent streptococci on the subsequent adhesion of *Candida albicans* to acrylic *in vitro*', J. Dent. 15, 73-76.

Weerkamp, A.H., Quirynen, M., Marechal, M., Van der Mei, H.C., Van Steenberghe, D. and Busscher, H.J. (1989) 'The role of surface free energy in the early *in vivo* formation of dental plaque on human enamel and polymeric substrata', Microbial Ecology in Health and Disease 2, 11-18.

ATTACHMENT IN INFECTION: THE INFLUENCE OF ENVIRONMENT ON STAPHYLOCOCCAL SURFACE PLASTICITY

S.P. DENYER*
Department of Pharmacy
Brighton Polytechnic
Moulsecoomb
Brighton BN2 4GJ, United Kingdom

M.C.DAVIES, J.A.EVANS-HURRELL & P.WILLIAMS
Department of Pharmaceutical Sciences
University of Nottingham, NG7 2RD,
United Kingdom

R.G. FINCH
Department of Microbial Diseases
City Hospital
Nottingham NG5 1PB, United Kingdom

* Correspondence

1. Introduction

Bacterial adherence to host tissue or indwelling medical devices is an important factor in the pathogenesis of many infections (Bisno and Waldvogel, 1989). Colonisation of such surfaces and subsequent biofilm formation affords protection against host defences, antimicrobial agents and other removal mechanisms (Williams, 1988). Furthermore, the establishment of a microenvironment serves to concentrate toxins and enzymes thereby encouraging local damage.

Peritonitis caused by coagulase-negative staphylococci is a major complication of continuous ambulatory peritoneal dialysis (CAPD; Bint et al., 1987). The dialysed cavity offers both medical device and peritoneal surfaces for potential colonisation and is a useful model by which to study the attachment process. Since this process is dependent on the nature of the surfaces involved, valuable information can be gained by examining the staphylococcal envelope in a suitable in vitro model.

Early studies have explored the adhesion of CAPD isolates following aerobic incubation in conventional laboratory media or used human peritoneal dialysate (HPD). Recent examination of used dialysis fluid obtained directly from patients revealed a high pCO_2 level which could not be sustained under aerobic incubation (Wilcox et al., 1990). In seeking to establish a suitable in vitro model, we have studied the influence of both medium and atmosphere on staphylococcal envelope character and adhesive properties. To judge the accuracy of our in vitro model we have attempted to correlate some envelope characteristics with those found in an animal peritoneal chamber implant model.

2. Methods

2.1 COLLECTION OF DIALYSATE

HPD was collected and pooled aseptically from the dialysed peritonea of 25 uninfected patients undergoing CAPD for the treatment of renal failure. Freedom from

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 355–361.

contaminating organisms and antibiotics was confirmed by sterility tests and diffusion assay. Dialysate composition is recorded in Wilcox et al. (1990).

2.2 BACTERIAL STRAINS AND CULTURE CONDITIONS

The three Staphylococcus epidermidis strains (designated 900, 901 and 904) were isolated from the dialysis fluid of CAPD patients with peritonitis attending the Renal Unit, City Hospital, Nottingham, UK. Staphylococci were speciated according to the API Staph system (API, Basingstoke, UK). Bacteria were cultured statically under an atmosphere of 5% CO_2 : 95% air at 37°C either in nutrient broth (Oxoid, Basingstoke, UK), in iron-restricted nutrient broth (achieved by the addition of 800μM EDDA), or in HPD, and subsequently harvested at late exponential/early stationary phase. For some studies with nutrient broth growing cells, a culture atmosphere of 100% air was employed. Where antibiotic-grown cells were required, each antibiotic was added to the appropriate medium, before inoculation, at a level of half the MIC. Cells were again harvested at the same phase of growth.

2.3 SURFACE ANALYSIS

Organisms were harvested from in vitro growth by centrifugation (5000 g for 30 minutes) and pellets washed and prepared for contact angle measurements, zeta potential determination and elemental and functional group analysis by X-ray photoelectron spectroscopy (XPS). A detailed treatment of the preparative stages and analytical methods used is given in Denyer et al. (1990).

2.4 CULTURE IN VIVO

Strain 901 was grown in vivo in a diffusion implant chamber described by Pike et al. (1991) and implanted intraperitoneally, in Wistar SBW rats (Modun et al., 1992). Cells were recovered after 48h implantation, collected and studied by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE; see 2.5 below) without further subculture.

2.5 PREPARATION OF CELL WALL AND CYTOPLASMIC MEMBRANE PROTEINS

Staphylococci were harvested from in vitro and in vivo growth and re-suspended in digestion buffer (Denyer et al., 1990). Protoplasts were removed by centrifugation, and the supernatant containing cell wall proteins was stored at -20°C until required. Cytoplasmic membranes were collected from subsequently lysed protoplasts. Proteins were extracted from wall and membrane preparations and subject to separation by SDS-PAGE (Denyer et al., 1990).

2.6 ADHERENCE STUDIES

A cover slip-based modification of the static overlay technique of Tavendale et al. (1983) was used. 1 ml aliquots of washed, in vitro grown bacterial cells were suspended in

Eagles Minimum Essential Medium with Earle's Salts (Gibco Europe Ltd, Paisley, Scotland, UK) and used to overlay HEp2 cell monolayers in multi-well compartments (Flow Laboratories Ltd, Irvine, Scotland, UK). After 90 minutes incubation at 37 °C in an atmosphere of 5% CO_2 : 95% air, unattached bacteria were removed by gentle washing, the monolayers fixed by 3% gluteraldehyde and stained by a modification of Grams method. Enumeration of attached bacteria was by microscopy and image analysis (Evans et al., 1990).

3. Results and Discussion

A superficial comparison based on gross staphylococcal surface physicochemistry would suggest that the growth environment has no major effect on the staphylococcal cell, although the three strains can be readily differentiated one from another by water contact angle determinations (Table 1).

TABLE 1. Physicochemical analysis of Staphylococcus epidermidis strains cultured in nutrient broth and human peritoneal dialysate

Growth medium	Strain	Physicochemical parameter	
		Zeta potential (mV)	Water contact angle (degrees)
Nutrient broth (100% air)	900	- 34	15
	901	- 40	27
	904	- 36	24
Nutrient broth (5% CO_2, 95% air)	900	- 37	15
	901	- 40	26
	904	- 36	24
Human peritoneal dialysate (5% CO_2, 95% air)	900	- 34	15
	901	- 40	28
	904	- 36	24

A more detailed examination of the surface chemistry, however, clearly revealed the profound effect of growth environment on elemental composition (Table 2).

TABLE 2. Surface elemental ratios of Staphylococcus epidermidis strains cultured in nutrient broth and in human peritoneal dialysate

Growth medium	Strain	Elemental Ratio		
		O/C	N/C	P/C
Nutrient broth (100% air)	900	0.360	0.180	0.039
	901	0.335	0.176	0.033
	904	0.345	0.167	0.021
Nutrient broth (5% CO_2, 95% air)	900	0.438	0.150	0.029
	901	0.384	0.198	0.034
	904	0.363	0.192	0.031
Human peritoneal dialysate (5% CO_2, 95% air)	900	0.404	0.190	0.051
	901	0.470	0.217	0.049
	904	0.414	0.207	0.042

In general, the elemental surface concentration ratios are in broad agreement with those described for six strains of staphylococci (van der Mei et al., 1989.) XPS suggested a significant increase in surface exposed carbohydrates and proteins (van der Mei et al., 1988) associated with staphylococcal culture in a CO_2- rich atmosphere and in HPD. The apparently raised amount of protein associated with strains grown in HPD may partially reflect the presence of some host-derived proteins as well as the expression of novel proteins (Williams et al., 1988). The detection of phosphorus would suggest the presence of surface lipoteichoic acid.

Confirmation of changes in cell envelope protein profiles was given by gel electrophoresis (Table 3). The similarity in protein expression between in vitro HPD-grown cells of strain 901 and those harvested from in vivo peritoneal culture is apparent, and the iron-dependency of the 32 and 36kDa membrane proteins can be inferred from this table.

TABLE 3. SDS-PAGE Protein expression and dominance in the envelope of Staphylococcus epidermidis (strain 901)

Growth medium	Cell wall	Cytoplasmic membrane
Nutrient broth (5% CO_2, 95% air)	Multiple bands; 39KDa predominant	Multiple bands; 41, 45 & 88kDa repressed
Iron-restricted nutrient broth (5% CO_2, 95% air)	Multiple bands; 39kDa repressed	Multiple bands; 32, 36 & 88kDa present
Human peritoneal dialysate (5% CO_2, 95% air)	Proteins significantly repressed including 39kDa; 42, 54 & 70kDa predominant	Some protein repression including 88kDa; 32, 36, 41 & 45 kDa dominant
In vivo peritoneal chamber implant	Proteins significantly repressed including 39kDa; 42, 54 & 70 kDa predominant	Some protein repression; 32, 36, 41, 45 and 88 kDa expressed

Under control conditions, the attachment profile of Staphylococcus epidermidis strains to HEp2 cells appears broadly similar under both nutrient broth and HPD culture in an atmosphere of 5% CO_2 : 95% air (Table 4). In the presence of a cefuroxime challenge, however, adhesive properties can be clearly contrasted between the two growth media implying a significantly different envelope sensitivity depending upon the growth environment.

TABLE 4. Adhesion of Staphylococcus epidermidis strains to HEp2 cells following culture in nutirient broth or human peritoneal dialysate in the presence and absence of 0.5 MIC antibiotic.

Growth medium	Strain	Adhesion (bacteria/HEp2 cell)		
		Control	Cefuroxime	Vancomycin
Nutrient broth	900	49	60	25
(5% CO_2, 95% air)	901	52	63	16
	904	19	17	11
Human peritoneal dialysate	900	38	22	26
(5% CO_2, 95% air)	901	37	13	16
	904	28	11	16

It is clear that the growth environment significantly affects the envelope character of Staphylococcus epidermidis and, most likely, surface related behaviour. There is evidence from our peritoneal chamber studies that in vitro culture in HPD under an atmosphere of 5% CO_2 : 95% air gives surface compositions more closely reflective of the in vivo situation. In the past, a disparate range of culture conditions have been employed in the laboratory study of microbial behaviour in CAPD-related peritonitis. Our studies indicate that the importance of growth environment in the development of in vitro models for the successful investigation of microbial adhesion and pathogenicity in CAPD-peritonitis cannot be overstated.

4. Acknowledgements

This work was supported by grants from the Wellcome Trust and Roussel Laboratories Ltd, UK.

REFERENCES

Bint, A.J., Finch, R.G., Gokal, R., Goldsmith, H.J., Junor, B. and Oliver, D. (1987). Diagnosis and managment of peritonitis in CAPD. Report of a working party of the British Society for Antimicrobial Chemotherapy. Lancet ii, 845-849.

Bisno, A.L. and Waldvogel, F.A. (Eds.) (1989). Infections associated with indwelling medical devices, ASM, Washington DC.

Denyer, S.P., Davies, M.C., Evans, J.A., Finch, R.G., Smith, D.G.E., Wilcox, M. and Williams, P.(1990). Influence of carbon dioxide on the surface characteristics and adherence potential of coagulase-negative staphylococci. Journal of Clinical Microbiology 28, 1813-1817.

Evans, J.A., Adler, J., Cheema, M.S. and Denyer, S.P. (1990). The use of image analysis to examine the effects of antibiotics on the adherence of Staphylococcus epidermidis to HEp2 cells. Journal of Pharmacy and Pharmacology 42, 102P.

Modun, B., Williams, P., Pike, W.J., Cockayne, A., Arbuthnott, J.P., Finch, R.G. and Denyer, S.P. (1992) cell envelope proteins of Staphylococcus epidermidis grown in vivo in a peritoneal chamber implant. Infection and Immunity 60(6), in press.

Pike, W.J., Cockayne, A., Webster, C.A., Slack, R.G.B., Shelton, A.P. and Arbuthnott, J.P. (1991). Development and design of a novel in vivo chamber implant for the analysis of microbial virulence and assessment of antimicrobial therapy. Microbial Pathogenesis 10, 443-450.

Tavendale, A., Jardine, C.K.H., Old, D.C.and Duguid, J.P. (1983) Haemaglutins and adhesion of Salmonella typhimurium to HEp2 and HeLa cells. Journal of Medical Microbiology 16, 371-380.

Van der Mei, H.C., Leonard, A.J., Weerkampa, H., Rouxhet, P.G. and Busscher, H.J. (1988) Properties of oral streptococci relevant for adherence : zeta potential, surface free energy and elemental composition. Colloids and Surfaces 32, 297-305.

Van der Mei, H.C., Brakke, P., Dankert, J., Feijen, J., Rouxhet, P.G. and Busscher, H.J. (1989). Physicochemical surface properties of non-encapsulated and encapsulated coagulase-negative staphylococci. Applied and Environmental Microbiology, 55, 2806-2814.

Wilcox, M.H., Smith, D.G.E., Evans, J.A., Denyer, S.P., Finch, R.G. and Williams, P. (1990) Influence of carbon dioxide on growth and antibiotic susceptibility of coagulase-negative staphylococci cultured in human peritoneal dialysate. Journal of Clinical Microbiology 28, 2183-2186.

Williams, P. (1988) Role of the cell envelope in bacterial adaptation to growth in vivo in infections. Biochimie 70, 987-1011.

Williams, P., Denyer, S.P. and Finch, R.G. (1988). Protein antigens of Staphylococcus epidermidis grown under iron-restricted conditions in human peritoneal dialysate. FEMS Microbiology Letters, 50, 29-33.

COAGULASE-NEGATIVE STAPHYLOCOCCI: ADHERENCE AND SLIME PRODUCTION

M.H. WILCOX
Department of Experimental and Clinical Microbiology,
University of Sheffield Medical School,
Beech Hill Road,
Sheffield S10 2RX
U.K.

1. Introduction

Coagulase-negative staphylococci (CNS) are now recognised as major nosocomial pathogens. Their isolation from clinical specimens continues to increase, in line with the widespread use of implantable medical devices. The most common manifestation of CNS infection is bacteraemia associated with a wide range of intravascular devices. In excess of 3500 peripheral and central intravascular catheters per year are now implanted into patients on one typical six-bedded intensive care unit (Royal Hallamshire Hospital, Sheffield, England). The average number of such devices per patient is greater than six. Other well recognised complications of implantable device-associated CNS infections include peritonitis in patients undergoing continuous ambulatory peritoneal dialysis (CAPD), endocarditis, mediastinitis, meningitis, and joint loosening and destruction.

2. Polymer surfaces

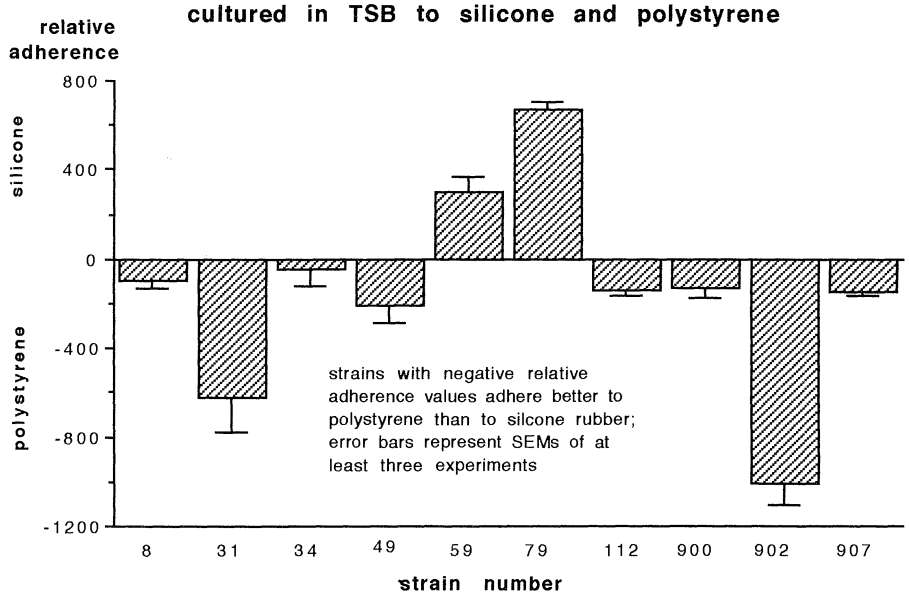
Many different materials have been used in the manufacture of implantable medical devices (Gristina et al., 1987; Hogt et al., 1987). These range from metals which are crystalline in structure, to the majority of medical polymers such as silicone, which are amorphous. Although the thrombogenic potential of polymer materials is considered when intravascular catheters are designed, the propensity for bacterial adherence is usually not addressed (Borrow et al., 1985). Studies have indicated that CNS appear to adhere initially to areas of polymer materials where the smooth surface is disrupted, probably because of favourable electrostatic forces here (Locci et al., 1981; Cheesbrough et al., 1985). However, some microscopically rough biopolymer surfaces such as hydrogel elastomers do not appear to be readily colonised by CNS (personal observations). Most in vitro studies of CNS adherence have used either glass or polystyrene surfaces, and yet neither of these materials are used to manufacture medical implants. Furthermore, the adherence of clinical CNS isolates to materials such as silicone rubber, which is used in many implants

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 363–370.

such as intravenous Hickman and CAPD catheters, is not predictable from the results of adherence assays using polystyrene (Figure 1) (Wilcox et al., 1991a).

Environmental factors such as growth rate, nutrient supply, temperature, pH, osmolarity and oxygen tension can each affect the structure and functon of the bacterial envelope in vitro (Brown et al., 1985). Also, culturing bacteria on solid media influences their surface composition; Cheung and Fischetti noted the induction of cell wall associated high molecular weight proteins in S. auerus grown on agar, compared to in broth (Cheung et al., 1988). The precise environmental conditions existing inside a mass of adherent cells and matrix remain uncertain, but these may be significantly altered from those experienced by planktonic bacteria. For example, local pH differences as great as 1.5 units can occur in the lower zones of biofilms growing on metallic surfaces (Costerton et al., 1987).

Figure 1 **Relative adherence of ten CNS isolates cultured in TSB to silicone and polystyrene**

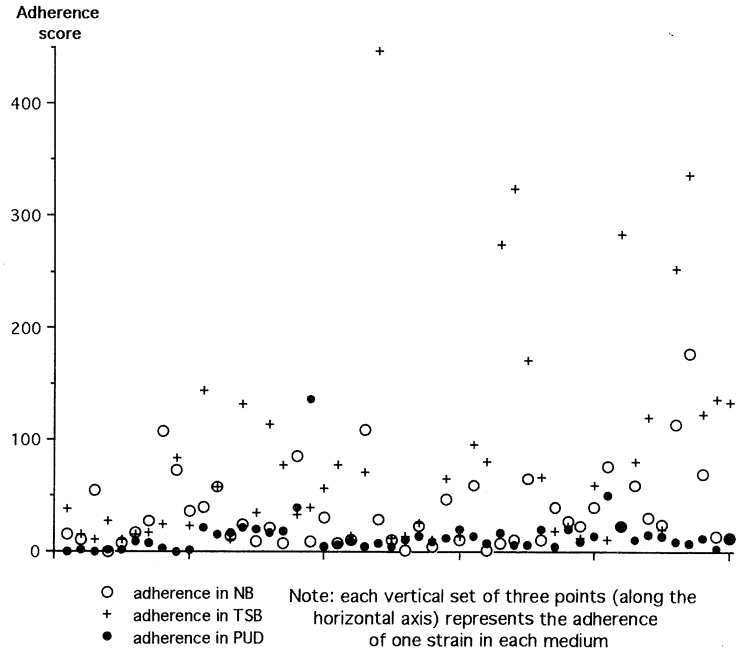


3. Gas tension during culture

Little attention has been given to the effects of gas tension on the surface composition of microbes. Changes in the envelope properties

All CNS species so far examined produce iron-repressible outer-membrane proteins when cultured in pooled used dialysis fluid (PUD), collected from patients undergoing CAPD (Smith et al., 1991; Wilcox et al., 1992). These proteins are immunogenic, being recognised by antibodies present in CAPD fluid and in patient serum. Other evidence of cell surface structural changes in biological fluids compared to conventional media, is provided by the marked shutdown in both cell wall and cell membrane proteins in SDS PAGE profiles of CNS grown in the former conditions (Smith et al., 1991). Any relationship of cell surface proteins, that are expressed in CNS cultured in media which reflect conditions found in vivo, to bacterial adherence potential has yet to be established. The adherent growth of CNS isolates is also markedly reduced in PUD compared to either nutrient broth (NB) or TSB, but the behaviour of individual strains is not predictable from results obtained in the latter complex media (Figure 2) (Hussain et al., 1992a). Albumin deposition onto surfaces immersed in biological fluids is known to reduce subsequent CNS adherence, and this may partly explain the gross reductions noted in media such as PUD (Peters et al., 1987). It is important to note that biological fluids such as PUD or human serum are buffered in vivo within a carbon dioxide/bicarbonate equilibrium. Physiological levels of carbon dioxide must be used in vitro when these fluids are employed as culture media, to maintain the pH and concentration of cations and proteins to those found in vivo.

Figure 2 **Comparison of adherent growth of 50 CNS strains to silicone rubber in three media in air with 5% CO2**



of Gram-negative bacteria grown under reduced redox potential have been reported (Maluszynksa et al., 1988). Also, Bayer et al recently showed that exopolysaccharide production by Ps. aeruginosa is oxygen dependent, and suggested this may account for the often refractory nature of left-sided pseudomonal endocarditis to aminoglycoside treatment (Bayer et al., 1989). They did not examine the influence of carbon dioxide (CO_2) levels on exopolysaccharide expression, however, despite a marked difference in the tension of this gas found on the two sides of the circulatory system. In vitro studies frequently involve culturing cells in air, but in doing so take no account of the possible influence of the increased CO_2 tensions found in vivo on bacterial behaviour.

We have demonstrated that if physiological levels of carbon dioxide (5% CO_2) are used during culture, the adherence of CNS is dramatically reduced compared to in air alone (Wilcox et al., 1991a). For example, mean reductions in adherent growth of 84% and 86% to silicone rubber and polystyrene respectively, were observed with 50 clinical CNS isolates cultured in tryptone soya broth (TSB). Occasional strains, however, consistently adhere better when cultured in air with 5% CO_2 than in air. A small reduction in pH (appoximately 0.2 units) occurs in CNS cultured in TSB in the CO_2 enriched atmosphere compared to in air, which may influence the surface charge of both bacteria and polymers. While no differences were found in the zeta potential or contact angle of Staphylococcus epidermidis strains cultured in these two atmospheres, X-ray photoelectric spectroscopy results (which allow an analysis of the surface elements and functional groups), indicate a significant increase in the surface exposure of both carbohydrates and proteins in bacteria grown in air with 5% CO_2 (Denyer et al., 1990). Cell wall protein profiles of S. epidermidis also demonstrate atmosphere-induced changes.

4. Nutrient supply: biological versus conventional media

In order to survive in vivo, microbes must adapt to the often severe limitation of nutrients that are found in tissues. However, despite increasing evidence of the important changes in envelope properties that occur in response to nutrient deprivation, cells are commonly cultured in conventional media which are replete in bacterial food substances (Brown et al., 1985). For example, under conditions of magnesium limitation some Gram-positive species increase the binding capacity of their cell walls for these metal cations, by producing more teichoic acid (Archibald, 1974). Bacterial exopolysaccharide synthesis has also been shown to be influenced by magnesium and calcium cations (Sutherland, 1983). Probably the best studied area of nutritional deprivation in bacteria is the effect of iron restriction on cell surface structure. The problem of iron acquisition is particularly acute for pathogenic bacteria in the peritoneal cavity of CAPD patients. Free iron is not detectable in either fresh or used dialysis fluid, in contrast to many other trace elements (Craddock et al., 1987).

Unless such measures are taken, the pH of PUD, for example, rises to approximately 8.5 in air, which will not support the growth of many CNS isolates (Wilcox et al., 1990).

Another prominent disadvantage of conventional complex media for CNS studies is the discovery that extracellular slime material recovered from these cells is contaminated by the growth medium. In particular, the high galactose content of some slime "preparations" previously reported has now been shown not to be of microbial origin, but instead to be derived from agar (Hussain et al., 1991a). Similarly, broth (TSB) grown bacteria produce slime, the constituents of which cannot be separated from those of the culture medium when column chromatography with DEAE cellulose is used (Hussain et al., 1992a). The advantages of using biological fluids thus once again became apparent. Chemically defined media (CDM) containing only low molecular weight substances, are a useful alternative (Hussain et al., 1991a; Hussain et al., 1991b). Clearly, high molecular weight material recovered from CNS cultured in CDM must be of microbial origin. Using this approach, Hussain et al have now proposed that CNS slime consists largely of cell wall teichoic acid (Hussain et al., 1992b).

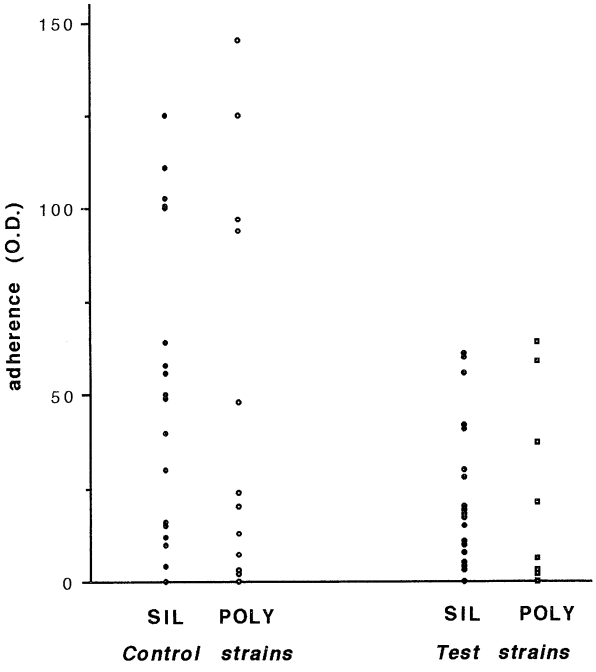
5. Slime production and adherence

The relationship of slime production and adherence has often been overlooked in studies of CNS pathogenicity. In particular, the fault of most in vitro assays of slime production is the reliance placed on initial bacterial adherence (often to clinically inappropriate polymers - see above) for a positive result. CNS strains that produce slime, but do not adhere to the surface in question are hence not recorded (Wilcox et al., 1991b). Others have proposed the use of lectins to detect extracellular slime. However, washed bacteria react with the same lectins as unwashed cells, and yet the slime is water soluble and is removed by such procedures. Peters et al noted that CNS first adhere to catheter surfaces and later produce slime (Peters et al., 1982). Using both scanning and transmission electron microscopy it is apparent that slime production and adherence are separate phenomena (Wilcox et al., 1991b). In addition to reduced CNS adherence in broth grown cells cultured in an atmosphere containing physiological levels of carbon dioxide, slime production is also grossly curtailed in such conditions. The potential of slime to consolidate surface microcolony formation, and to protect CNS within a biofilm has still to be clarified.

Two hypotheses concerning the pathogenesis of CNS in relation to polymer surface colonisation may be put forward. Firstly, strains that possess virulence factors such as the ability to adhere and/or to produce slime are at a survival advantage, and so can prosper on medical implants. Several studies have indicated that slime production, (in fact, adherent growth - see above), is significantly more common amongst CNS isolates from patients with infected prostheses than in controls (usually, either blood culture contaminants or skin

commensals) (Davenport et al., 1986; Younger et al., 1987). Other groups have failed to find such an association (Needham et al., 1984). However, these reports concern broth grown bacteria, polystyrene or glass surfaces, and atmosphere conditions not found in vivo. A recent study in this department has compared the adherence to silicone rubber of 22 CNS blood culture isolates from Hickman line infections, with that of 22 CNS recovered from the hands of volunteers. The proportions of S. epidermidis strains in each group were matched. Culture was performedin pooled human serum at 37°C for 48 hours, with fresh serum being introduced after 24 hours, in an atmosphere containing 10% oxygen and 5% CO_2 (venous blood levels). No significant difference in the adherent growth of these groups of isolates was demonstrated (Figure 3). These results suggest a second hypothesis, namely that all CNS have the ability to cause implant associated infection if they are introduced into these settings. Further studies are underway to confrm the latter hypothesis, which has implications for the additional care that should be taken to ensure that aseptic techniques are adhered to, if CNS device associated infection is to be prevented.

Figure 3 **Adherent growth of blood culture CNS isolates (n=22) compared to control skin isolates (n=22), in serum in a physiological atmosphere to both silicone rubber (SIL) and polystyrene (POLY)**



REFERENCES

Archibald, A.R. (1974) The structure, biosynthesis and function of teichoic acids. Adv Microb Physiol, 11, 53-95.

Bayer, A.S., O'Brien, T., Norman, D.C., Nast, C.C. (1989) Oxygen-dependent differences in exopolysaccharide production and aminoglycoside inhibitory-bactericidal interactions with *Pseudomonas aeruginosa* - implications for endocarditis. J Antimicrob Chemother, 23, 21-35.

Borrow, M., Crowley, J.G. (1985) Evaluation of central venous catheter thrombogenicity. Acta Anaesthesiol Scand (suppl), 81, 59-64.

Brown, M.R.W., Williams, P. (1985) The influence of environment on envelope properties affecting survival of bacteria in infections. Ann Rev Microbiol, 39, 527-556.

Cheesbrough, J.S., Elliott, T.S.J., Finch, R.G. (1985) A morphological study of bacterial colonisation of intravenous cannulae. J Med Microbiol, 19, 149-157.

Cheung, A.L., Fischetti, V.A. (1988) Variation in the expression of cell wall proteins of *Staphylococcus aureus* grown on solid and liquid media. Infect Immun, 56,1061-1065.

Costerton, J.W., Geesey, G.G., Jones, P.A. (1987) Bacterial biofilms in relation to internal corrosion, monitoring and biocide strategies. Corrosion 87, San Francisco, pp. 1-9.

Craddock, C.F., Edwards, R., Finch, R.G. (1987) Pseudomonas peritonitis in continuous ambulatory peritoneal dialysis: laboratory predictors of treatment failure. J Hosp Infect, 10, 179-186.

Davenport, D.S., Massanari, R.M., Pfaller, M.A., Bale, M.J., Streed, S.A., Hierholzer, W.J. (1986) Usefulness of a test for slime production as a marker for clinically significant infections with coagulase-negative staphylococci. J Infect Dis, 153, 332-339.

Denyer, S.P., Davies, M.C., Evans, J.A., Finch, R.G., Smith, D.G.E., Wilcox, M.H., Williams, P. (1990) Influence of carbon dioxide on the surface characteristics and adherence potential of coagulase-negative staphylococci. J Clin Microbiol, 28, 1813-7.

Gristina, A.G., Hobgood, C.D., Barth, E. (1987) Biomaterial specificity, molecular mechanisms and clinical relevance of *S. epidermidis* and *S. aureus* infections in surgery. In: Pulverer G., Quie P.G., Peters G., eds. Pathogenicity and clinical significance of coagulase-negative staphylococci. Zbl Bakt Suppl 16. Stuttgart. Gustav Fischer Verlag, pp. 143-157.

Hogt, A.H., Dankert, J., Feijen, J. (1987) Adhesion of staphylococci onto biomaterials. In: Pulverer G., Quie P.G., Peters G. eds. Pathogenicity and clinical significance of coagulase-negative staphylococci. Zbl Bakt Suppl 16. Stuttgart. Gustav Fischer Verlag, pp. 113-131.

Hussain, M., Hastings, J.G.M., White, P.J. (1991b) A chemically defined medium for slime production by coagulase-negative staphylococci. J Med Mirobiol, 34, 143-147.

Hussain, M., Hastings, J.G.M., White, P.J. (1991a) Isolation and composition of the extracellular slime made by coagulase-negative staphylococci in a chemically defined medium. J Infect Dis, 163, 534-541.

Hussain, M., Hastings, J.G.M., White, P.J. (1992b) Comparison of wall teichoic acid with high molecular weight extracellular material (slime) from *Staphylococcus epidermidis*. J Med Microbiol. In press.

Hussain, M., Wilcox, M.H., White, P.J., Faulkner, M.K.,Spencer, R.C. (1992a) Importance of medium and atmosphere type to both slime production and adherence by coagulase-negative staphylococci. J Hosp Infect, 20, 173-184.

Locci, R., Peters, G., Pulverer, G. (1981) Microbial colonisation of prosthetic devices. III Adhesion of staphylococci to lumina of intravenous catheters perfused with bacterial suspensions. Zbl Bakt Hyg B, 173, 300-302.

Maluszynska, G.M., Magnusson, K.E., Stendahl, O., Lock, R., Kniola, B. (1988) Reduced redox potential during growth of some gram-negative bacteria. APMIS, 96, 773-782.

Needham, C.A., Stempsey, W. (1984) Incidence, adherence and antibiotic resistance of coagulase-negative *Staphylococcus* species causing human disease. Diagn Microbiol Infect Dis, 2, 293-299.

Peters, G., Locci, R., Pulverer, G. (1982) Adherence and growth of coagulase-negative staphylococci on surfaces of intravenous catheters. J Infect Dis, 146, 479-482.

Peters, G., Schumacher-Perdreau, F., Jansen, B., Bey, M., Pulverer, G. (1987) Biology of *S. epidermidis* extracellular slime. In: Pulverer G., Quie P.G., Peters G, eds. Pathogenicity and clinical significance of coagulase-negative staphylococci. Zbl Bakt Suppl 16. Stuttgart. Gustav Verlag, pp. 15-31.

Smith, D.G.E.,Wilcox, M.H., Finch, R.G., Denyer, S.P., Williams, P. (1991) Characterisation of the cell envelope proteins of *Staphylococcus epidermidis* cultured in human peritoneal dialysate. Infect Immun, 59, 617-24.

Sutherland, J.W. (1983) Microbial exopolysaccharides - their role in microbial adhesion in aqueous systems. CRC Crit Rev Microbiol, 10, 173-201.

Wilcox, M.H., Denyer, S.P., Finch, R.G., Smith, D.G.E., Williams, P. (1990) Influence of carbon dioxide on the growth and antibiotic sensitivity of coagulase-negative staphylococci cultured in human peritoneal dialysate. J Clin Microbiol, 28, 2183-6.

Wilcox, M.H., Denyer ,S.P., Finch, R.G., Smith, D.G.E., Williams, P. (1991a) Effects of carbon dioxide and sub-lethal levels of antibiotics on adherence of coagulase-negative staphyloccoci to polystyrene and silicone rubber. J Antimicrob Chemother, 27, 577-87.

Wilcox, M.H., Hussain, M., Faulkner, M.K., White, P.J., Spencer, R.C. (1991b) Slime production and adherence by coagulase-negative staphylococci. J Hosp Infect, 18, 327-32.

Wilcox, M.H., Williams, P., Smith, D.G.E., Modun, B., Finch, R.G., Denyer, S.P. (1992) Variation in the expression of cell envelope proteins of coagulase-negative staphylococci cultured under iron-restricted conditions in human peritoneal dialysate. J Gen Microbiol, 137, 2561-70.

Younger, J.J., Christensen, G.D., Bartley, D.L., Simmons, J.C.H., Barrett, F.F. (1987) Coagulase-negative staphylococci isolated from cerebrospinal fluid shunts: importance of slime production, species identification and shunt removal to clinical outcome. J Infect Dis, 156, 548-554.

POLYSACCHARIDE INTERACTIONS IN BACTERIAL BIOFILMS

D. G. ALLISON
Pharmacy Department
Manchester University
Manchester M13 9PL
England

1. Introduction

Biofilms may be considered as a highly structured functional consortia of cells, attached to substrata within extracellular polysaccharide (EPS) containing matrices (Costerton *et al*, 1987). Their physiology, metabolism and organisation is greatly dependent upon the nature of those substrata and also upon the prevailing physicochemical environment. In many instances, the problematic nature of the presence of microorganisms is compounded by the recalcitrance of such populations to treatment with chemical antimicrobial agents and antibiotics (Brown *et al*, 1988;Gilbert *et al*, 1990). This is particularly true in patients with fibrocystic lung disease (CF), where chronic lung infection by mucoid strains of *Pseudomonas aeruginosa* is associated with progressive pulmonary infection and mortality (Hoiby, 1974). Under such conditions the organism is often found growing slowly, under iron limitation (Anwar *et al*, 1984) as a biofilm (Costerton *et al*, 1987). The severity of lung infection in patients colonised with *P. aeruginosa* has been directly correlated with the production of a highly viscous, alginate-like EPS (Gordon *et al*, 1988). In this respect, difficulty in eradicating *P. aeruginosa* from the lungs has been attributed to retarded antibiotic penetration through the EPS matrix associated with the biofilm.

The viscosity and gel-forming characteristics of alginate are influenced by the molecular weight and polyanionic nature of the EPS (Russel & Gacesa, 1988). As such, alginate gelation is induced by the presence of divalent cations, particularly calcium. This has been shown to decrease the rate of aminoglycoside diffusion through such gels (Gordon *et al*, 1988) due to an increase in cross-linking within the polymer. The clinical relevance of such studies is questionable since alginates isolated from the CF lung show considerable compositional heterogeneity. Morover, *P. aeruginosa* has been shown to co-synthesise a discrete low molecular weight EPS (LMW.EPS) under certain growth conditions (Allison *et al*, 1991). In addition, *Pseudomonas cepacia* has recently emerged as an important pathogen in CF which can co-inhabit the lung with *P. aeruginosa* (Gilligan, 1991). *P. cepacia* also has the ability to produce large amounts of EPS. Hence, characteristic to the CF lung is the overproduction of mucous by the host and the secretion of different bacterial EPS. The purpose of the present study was to determine the rheological nature of EPS interactions occurring between different bacterial and host polysaccharides and to assess the effect upon antibiotic penetration.

2. Materials and Methods

Bacteria and Culture Conditions.

P. aeruginosa strain PaWH (mucoid), isolated from the sputum of a CF patient and *P. cepacia* NCTC

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 371–376.

10661 were grown at 37°C on yeast extract (YE) media as previously described (Allison & Sutherland, 1987). Stock cultures were maintained at -20°C in nutrient broth containing 10% glycerol.

EPS EXTRACTION AND PURIFICATION.

EPS was isolated from cell suspensions according to the methods described by Allison and Matthews (in press). The methods of Kennedy and Sutherland (1987) were used to determine EPS composition.

VISCOSITY DETERMINATIONS.

EPS viscosity was determined for i) purified polymers of *P. aeruginosa*, *P. cepacia* and commercially available mucin (sigma) at various concentrations and ii) a mixture of all polymers plus calcium ions using a simple U-tube capillary viscometer designed for small volume samples. The time taken (s^{-1}) for the sample to fall a fixed distance under gravity at 35°C was taken as a measure of relative viscosity. Measurements were repeated in triplicate to an accuracy of $0.5s^{-1}$.

ANTIBIOTIC SUSCEPTIBILITY TESTING.

Preliminary experiments, utilising mid-log batch culture of the test organism, were conducted to establish those concentrations of tobramycin ($20\mu gml^{-1}$) and ciprofloxacin ($0.5\mu gml^{-1}$) which gave appropriate levels of killing (1-2 log cycles) within a 1h contact period at 35°C. Levels of survival in all cases decreased with increasing drug concentration. The concentrations were used subsequently to assess the effect of EPS rheological interactions on antibiotic penetration. Appropriate controls were incorporated into each experiment to circumvent antibiotic/ionic effects. To assess the rate of killing, a dialysis bag (6000 Mol. wt. cut off) containing either antibiotic (1ml) or antibiotic plus EPS solution (1ml) was aseptically added to a 250ml erlenmeyer flask containing 100ml *P. cepacia* cell suspension resuspended to a concentration of 5×10^7 cells.ml^{-1} in YE salts. Samples were removed at regular intervals, serially diluted in sterile normal saline and viable counts made onto the surfaces of pre-dried nutrient agar plates in triplicate. All plates were subsequently incubated for 16h at 35°C. Results were expressed as percentage reductions in viability relative to appropriate unexposed controls.

3. Results

EPS BIOSYNTHESIS AND CHARACTERISATION.

Previous studies by Allison *et al* (1991) have shown *P. aeruginosa* to produce both a high molecular weight alginate-like polysaccharide and a chemically distinct low molecular weight polymer only when grown as a biofilm population. This LMW.EPS has a molecular mass of approximately 10^4 and is composed of galactose, mannose and an as yet unidentified aminosugar in a 2:1:1 molar ratio. Although the precise role of this LMW.EPS is unknown, preliminary studies have demonstrated an involvement in cell adhesion (unpublished results).

The EPS produced by *P. cepacia* had an approximate molecular mass of between 4×10^5 and 9×10^5 when isolated from either agar or broth grown cultures respectively (Table 1). There was no evidence of a LMW.EPS being produced by cells grown on a solid surface. However, variations in EPS composition were noted, dependent upon the mode of growth (Table 1). In this respect, although the molar ratio of the galactose, glucose, mannose and glucuronic acid residues was identical for agar and broth cultures, the amount of rhamnose present was significantly different for the two cultures. This

would imply the EPS to possess a rhamnose side group. In addition, acetate and pyruvate content varied with culture conditions. On agar culture there was a 3.5 fold decrease in pyruvate content yet a 4-fold increase in acetate. Such variations, particularly in acetate concentration, can have a significant influence on the rheological properties of polysaccharides (Sutherland, 1990).

TABLE 1. Composition of *P. cepacia* EPS isolated from agar and broth grown cultures.

	Carbohydrate Composition (molar ratio)					Non-Carbohydrate Composition (%)		Molecular Mass
	Gal:	Glu:	Man:	GlcUA:	Rha	Acetate	Pyruvate	
Agar	1	1	2	1	0.2	8.1	4.3	4×10^5
Broth	1	1	2	1	0.5	2.2	14.2	9×10^5

VISCOSITY MEASUREMENTS.

Relative viscosity measurements for the different polysaccharides are shown in Figure 1. It is evident that both the alginate and the EPS obtained from *P. cepacia* possessed similar rheological properties, increasing in viscosity with polysaccharide concentration. The LMW.EPS derived from *P. aeruginosa* demonstrated a similar response, but at a much reduced viscosity. Mucin on the otherhand, showed a different response. Whilst a low viscosity similar to that of alginate and *P. cepacia* EPS was measured for a 0.1% solution, increasing the concentration of polysaccharide resulted in an exponential increase in mucin viscosity. When all four polymers were combined, in equal amounts, a marked increase in relative viscosity was observed. This effect was dramatically heightened by the addition of 3mM calcium ions to the combined polymeric solution. The same Ca^{2+} induced effect was not observed with any one individual polymer but was dependent upon the presence of alginate, mucin and *P. cepacia* EPS.

ANTIBIOTIC SENSITIVITY.

Figure 2 illustrates the effect of polysaccharide viscosity on the bactericidal properties of tobramycin and ciprofloxacin against *P. cepacia*. After 5 hours incubation in the presence of tobramycin, *ca.* 98% of the control population was killed. In contrast, when tobramycin was added to the combined polymer\Ca^{2+} solution, the population was reduced by only 10% after a similar period. Such results are not totally unexpected since the anionic bacterial polysaccharides will bind and retard the diffusion of the cationic aminoglycoside. Surprisingly, ciprofloxacin activity was also affected. At a concentration of $0.5\mu gml^{-1}$, *ca.* 75% of the control population was killed after 5h incubation, whereas in the presence of the combined polymeric mixture, the number of survivors increased to 60%.

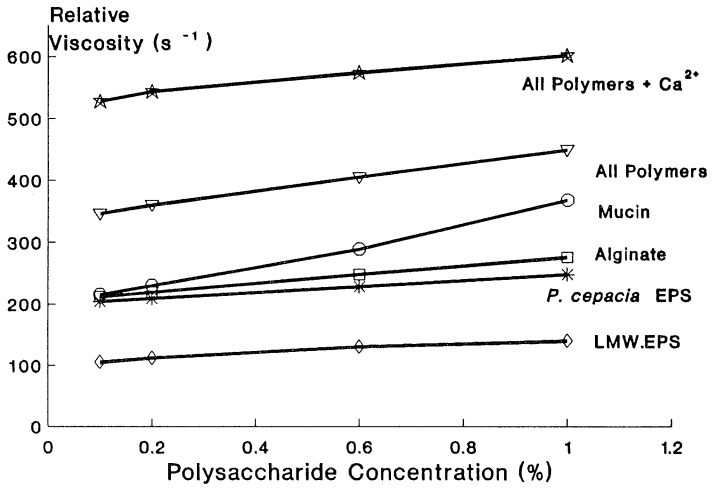


Figure 1. Polysaccharide viscosity as a function of concentration.

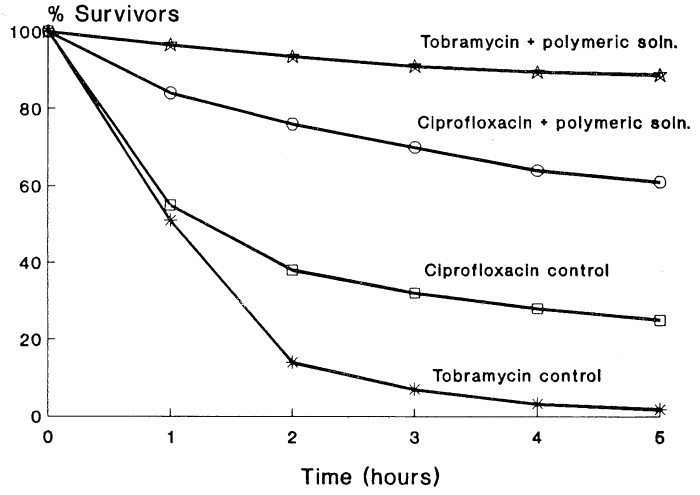


Figure 2. Effect of polysaccharide gellation on antibiotic activity.

Discussion

It is well established that the structure and composition of the Gram-negative cell envelope changes markedly in response to changes in the surrounding environment (Brown & Williams, 1985; Gilbert *et al*, 1990). Adherent populations of microorganisms differ from planktonic cells with respect to many important surface features (Allison *et al*, 1990) and can adopt phenotypes, characteristic to those environments, which can be particularly recalcitrant to antimicrobial agents (Brown *et al*, 1988; Gilbert *et al*, 1990). In CF, the selection and establishment of mucoid strains of *Pseudomonas* attached to the lung mucosa ultimately leads to mortality in such patients. In addition to the synthesis of a high molecular weight alginate-like polysaccharide, *P. aeruginosa* has also been shown to produce a chemically distinct LMW.EPS. This second polymer is only synthesised when grown in the presence of a solid surface. Moreover, preliminary studies have indicated this polymer to be involved in the attachment of *P. aeruginosa* to surfaces and in the synergistic attachment of *P. cepacia* (unpublished results).

The EPS produced by *P. cepacia* is also subject to environmental modulation with respect to its composition and molecular mass (Table 1). Of particular significance is the variation in rhamnose content, indicative of the polymer containing a side-chain, and the level of acetate. As the acetate concentration increases, the lipophilicity of the polymer will be increased, thereby affecting the capacity to interact with other polysaccharides and cations. In addition, the strength of interaction between two or more polysaccharides is influenced by side chain components (Sutherland, 1990). Such factors are likely to be extremely significant in the CF lung where different bacterial and host polysaccharides are secreted in the same microenvironment.

Antibiotic resistance in CF infections has been largely attributed to failure to penetrate the EPS matrix associated with the biofilm (Gordon *et al*, 1988; Nichols *et al*, 1989). This is particularly true for the aminoglycosides and to a limited extent, ß-lactam antibiotics. The results presented in Figure 2 also demonstrate ciprofloxacin activity to be markedly reduced due to interactions with the polysaccharide complex. As such, although ciprofloxacin has been effective in the therapy of other microbial chest infections, it is unable to eradicate either *P. aeruginosa* or *P. cepacia* from the lungs of CF patients (Fass, 1987). Assessment of the clinical significance, however, is complicated by the variability in composition of alginate, both within and between *P. aeruginosa* strains (Pugashetti *et al*, 1987) and by the lack of data describing the concentration of EPS and size of the microcolonies *in vivo*. Moreover, whilst studies utilising homodisperse polysaccharide solutions have their value, they do not reflect the interactions occurring *in vivo* within heterodisperse polysaccharide solutions. The results presented here clearly demonstrate a substantial increase in viscosity for a combined solution of bacterial and host polymers in comparison to individual polysaccharides (Fig 1). This rise in viscosity is further increased by the addition of Ca^{2+} ions. Such conditions are typical of those found in the CF lung. Thus, although the use of mucolytic and slime dispersal agents have been shown to promote antibiotic diffusion through alginate (Gordon *et al*, 1991), the use of such agents on heterodisperse polysaccharide solutions has received no attention. Since the majority of bacterial biofilms are composed of a heterogeneous population, irrespective of the environment, a consideration of the rheological interactions is merited.

References

Allison, D.G. & Sutherland, I.W. (1987). 'The role of exopolysaccharides in adhesion of freshwater bacteria', J. Gen. Microbiol. **133**, 1319-1327.

Allison, D.G., Evans, D.J., Brown, M.R.W. & Gilbert, P. (1990). ' Surface hydrophobicity of *Pseudomonas aeruginosa* biofilms cultured at various specific growth rates', FEMS Microbiol. Lett. **71**, 101-104.

Allison, D.G., Brown, M.R.W. & Gilbert, P. (1991). 'Slow adherent growth modulates polysaccharide

production by *Pseudomonas aeruginosa*', Biofouling, **4**, 243.

Allison, D.G. & Matthews, M.J. 'Effect of polysaccharide interactions on antibiotic susceptibility of *Pseudomonas aeruginosa*', J.Appl.Bacteriol **In Press**.

Anwar, H., Brown, M.R.W., Day, A. & Weller, P.H. (1984). 'Outer membrane antigens of mucoid *Pseudomonas aeruginosa* isolated directly from the sputum of a cystic fibrosis patient', FEMS Microbiol. Lett. **24**, 235-239.

Brown, M.R.W. & Williams, P. (1985). 'The influence of the environment on envelope properties affecting survival of bacteria in infections', Ann.Rev.Microbiol. **39**, 527-556.

Brown, M.R.W., Allison, D.G. & Gilbert, P. (1988). 'Resistance of bacterial biofilms to antibiotics: a growth-rate related effect?',J.Antimicrob.Chemother. **22**, 777-780.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C. *et al* (1987). 'Bacterial biofilms in nature and disease', Ann.Rev.Microbiol. **41**, 435-464.

Fass, R.J. (1987). 'Efficacy and safety of oral ciprofloxacin in the treatment of serious respiratory infections', Am.J.Med. **82**, *suppl. 4A*, 202-207.

Gilbert, P., Collier, P.J. & Brown, M.R.W. (1990). 'Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy and stringent response', Antimicrob. Agents chemother. **34**, 1865-1886.

Gilligan, P.H. (1991). 'Microbiology of airway disease in patients with cystic fibrosis', Clin. Microbiol. Rev. **4**, 35-51.

Gordon, C.A., Hodges, N.A. & Marriott, C. (1988). 'Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis-derived *Pseudomonas aeruginosa*', J.Antimicrob.Chemother. **22**, 667-674.

Gordon, C.A., Hodges, N.A. & Marriott, C. (1991). 'Use of slime dispersants to promote antibiotic penetration through the extracellular polysaccharide of mucoid *Pseudomonas aeruginosa*', Antimicrob. Agents Chemother. **35**, 1258-1260.

Hoiby, N. (1974). '*Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral response', Acta.Path.Microbiol.Scand., *Section B*, **82**, 551-558.

Kennedy, A.F.D. & Sutherland, I.W. (1987). 'Analysis of bacterial exopolysaccharides', Biotech.Appl.Biochem. **9**, 12-19.

Nichols, W.W., Evans, M.J., Slack, M.P.E. & Walmsley, H.L. (1989). 'The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*', J.Gen.Microbiol. **135**, 1291-1303.

Pugashetti, B.K., Metzger, H.M., Vadas, L. & Feingold, D.S. (1987). 'Phenotypic differences among clinically isolated mucoid *Pseudomonas aeruginosa* strains', J.Clin.Microbiol. **16**, 686-691.

Russel, N.J. & Gacesa, P. (1988). 'Chemistry and biology of mucoid strains of *Pseudomonas aeruginosa* in cystic fibrosis', Mol.Aspects Med. **10**, 1-91.

Sutherland, I.W. (1990). 'Biotechnology of microbial exopolysaccharides', in J.Baddiley, N.H. Carey, I.J. Higgins & W.G.Potter (eds), Cambridge studies in biotechnology, Cambridge University press, pp 12-117.

Chapter 6

BIOFILMS IN FOOD PROCESSING

IMPLICATIONS OF ENGINEERING DESIGN IN FOOD INDUSTRY HYGIENE

D.A. TIMPERLEY, R.H. THORPE AND J.T. HOLAH
Campden Food and Drink Research Association
Chipping Campden
Glos GL55 6LD
United Kingdom

1. Introduction

Of the environmental contamination routes in food processing, surfaces are probably the most important route of infection both directly via product contact surfaces (production equipment) or indirectly as reservoirs of infection for product contamination via the other routes (e.g. drains, floors, washroom surfaces). Fundamental to the control of contamination of equipment is hygienic design. Good hygienic design prevents the retention of product out of the main flow during processing or product soils (including microorganisms) after cleaning procedures. Poor hygienic design is often characterised by rough surfaces, crevices and dead spaces which can retain product residues. Equipment which retains product residues after cleaning cannot generally be decontaminated and, therefore, contamination cannot be controlled.

This paper describes, in relation to current developments in the field, the fundamentals of food equipment hygienic design, how it may be assessed and how material surfaces may change with wear. Whilst being of particular concern to the food industry in terms of the deleterious effects that can arise from a 'small' number of microorganisms remaining on equipment after cleaning, it is hoped that the principles established in the food industry may be applicable to other industrial sectors where control of microorganisms is required.

2. Hygienic Design

Food processing equipment must not only perform the function for which it has been designed, but also it must do so hygienically. Hygienic design is that aspect of design which will ensure that the equipment will not result in the production of food which may adversely affect the health of the consumer and, after use, it can be restored to an acceptable level of cleanliness, economically and in a reasonable time.

Hygienic design has received some attention in the past and a number of useful texts and guidelines have been published (Jowitt 1980, Anon 1982a, 1982b, 1983a, 1987, Thorpe & Barker 1985, 1987, Timperley *et al.* 1990, 1992). A number of countries have national standards and/or directives applicable to the hygienic design of food machinery but there are relatively few international standards and these are predominantly dairy based (Anon 1973, 1974, 1976, 1980, 1982,

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 379–393.

1983a, 1983b, 1987). These standards/directives are too general, however, and are based on 'experience' rather than scientific data. Further to this, in the USA, a number of guidelines in the form of third party approval schemes have been developed for the dairy industry (the 3-A standards) and food service equipment (the National Sanitation Foundation (NSF)). The structure of these schemes is commendable in that representatives of equipment manufacturers, users and regulatory bodies are all involved in the implementation of recommendations. Unfortunately, however, the 3-A Standards have no bench mark of cleanability or test regimes to establish cleanability, and the NSF Standards are not applicable to the hygienic design of general food processing equipment.

In the EC, the Council Directive on the approximation of the laws of Member States relating to machinery (89/392/EEC) was published on the 14th June 1989 and is predominantly concerned with safety aspects. However, the Directive includes a short section dealing with hygiene and design requirements in Annex I, Section 2.1 - Agri-foodstuffs machinery. Section 2.1 states that machinery intended for the preparation and processing of foods must be designed and constructed so as to avoid health risks and consists of seven hygiene rules that must be observed. These rules are concerned with the suitability and cleanability of materials in contact with food; surface finish and design features such as joints, absence of ridges and crevices; avoidance of the use of fasteners, e.g. screws and rivets; the design of internal angles and corners; drainage of residues from equipment surfaces; dead spaces and voids, and lastly bearings and shaft seals. In addition to these hygiene rules, Section 2.1 contains a requirement that machinery manufacturers must indicate the recommended products (chemicals) and methods for cleaning, disinfecting and rinsing both open equipment, e.g. a conveyor, and closed equipment, e.g. pipelines, valves and pumps, where clean-in-place (CIP) procedures need to be used.

The Directive requires that all machinery sold within the EC after 1992 shall meet certain basic standards. The Commission turned to the European Standard Organisation (CEN) to produce these standards for both safety and hygiene and CEN has set up a series of technical committees to carry out the required work. The Chief Technical Committee for Machinery Standards is CEN/TC 114. Below this there are a series of sub-committees, all of which have the standing of a technical committee. CEN/TC 153 is concerned with food processing machinery, safety and hygiene specifications. The committee has responsibility for 17 groups of industrial equipment which are listed in Appendix 1. Food equipment for domestic use is not included.

Technical Committee CEN/TC 153 has set up two ad hoc working groups to prepare horizontal (Category A) standards on the basic concepts for hygiene requirements and safety requirements, respectively. The remit of the Ad hoc Working Group on Hygiene Requirements is to prepare a Draft Standard on Hygiene Requirements which is applicable to the 17 groups of food equipment covered by CEN/TC 153 - that is, to present the principles of hygienic design which apply to all food preparation and processing equipment, irrespective of the type or throughput of the machine or the product concerned as stated in Annex I, Section 2.1 of the Directive.

The basic hygienic design requirements can be presented under nine headings:-

2.1 MATERIALS OF CONSTRUCTION

Materials used for product contact must have adequate strength over a wide temperature range, a reasonable life, be non-tainting, corrosion and abrasion

resistant, easily cleaned and capable of being shaped. Stainless steel usually meets all these requirements which is why it is still the most widely used material of construction. There are various grades of stainless steel which are selected for their particular properties to meet operational requirements, e.g. Type 316 which contains molybdenum is used where improved corrosion resistance is necessary.

2.2 SURFACE FINISH

Product contact surfaces must be finished to a degree of surface roughness that is smooth enough to enable them to be easily cleaned. There are instruments for measuring surface texture on a numerical basis and the internationally recognised roughness value is designated μm R_a. In addition, a profile graph of a surface can also be provided by these instruments which allows examination to be made of the shape, height and spacing of the surface irregularities.

2.3 JOINTS

Permanent joints, such as those which are welded, should be smooth and continuous. Even if the welds of the lap joint shown in Fig. 1 were to be ground and polished, there would still be a step which could result in cleaning problems. Butt welding, as shown, will result in a smooth, continuous surface provided that the joint is ground and polished to the same standard as the surrounding material.

Dismountable joints, such as screwed pipe couplings shown in Figs. 2, 3 and 4, must be crevice-free and provide a smooth continuous surface on the product side. Flanged joints, as shown in Fig. 5, must be located with each other and be sealed with a gasket because although metal/metal joints can be made leak tight they may still permit the ingress of microorganisms.

2.4 FASTENERS

Exposed screw threads, Fig. 6, nuts, bolts, screws and rivets must be avoided wherever possible from product contact areas. Alternative methods of fastening can be used, as shown.

2.5 DRAINAGE

All pipelines and equipment should be self draining, as shown in Figs. 7 and 8, because residual liquids can result in contamination of product.

2.6 INTERNAL ANGLES AND CORNERS

These should be well radiused, wherever possible, to facilitate cleaning, as shown in Fig. 9.

2.7 DEAD SPACES

As well as ensuring that there are no dead spaces in the design of equipment,

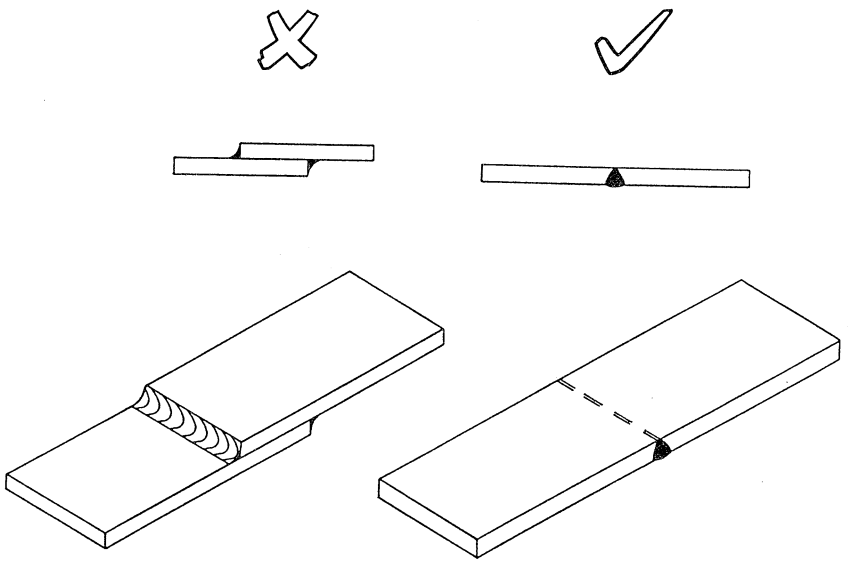


Figure 1. Welded joints

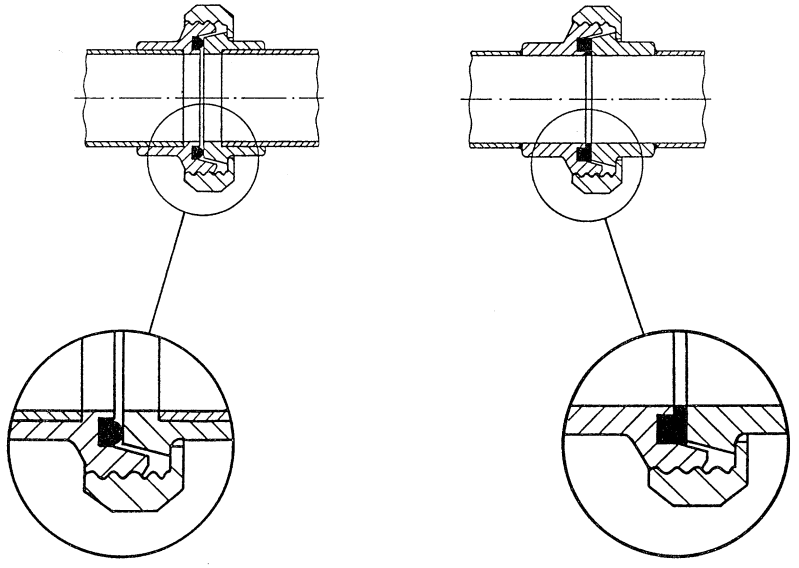


Figure 2. DIN 11851 screwed pipe coupling

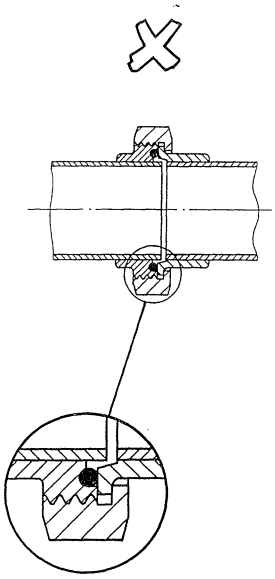


Figure 3. BS 1864 RJT
Screwed pipe coupling

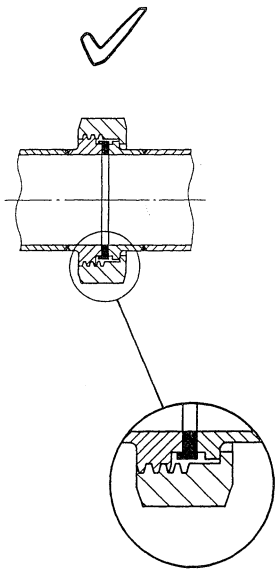


Figure 4. ISO 2853/BS 4825
Part 4 Screwed pipe coupling

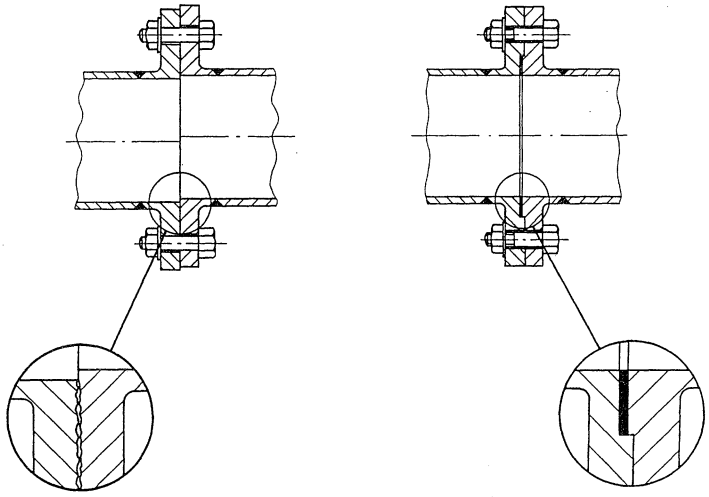


Figure 5. Flanged joint

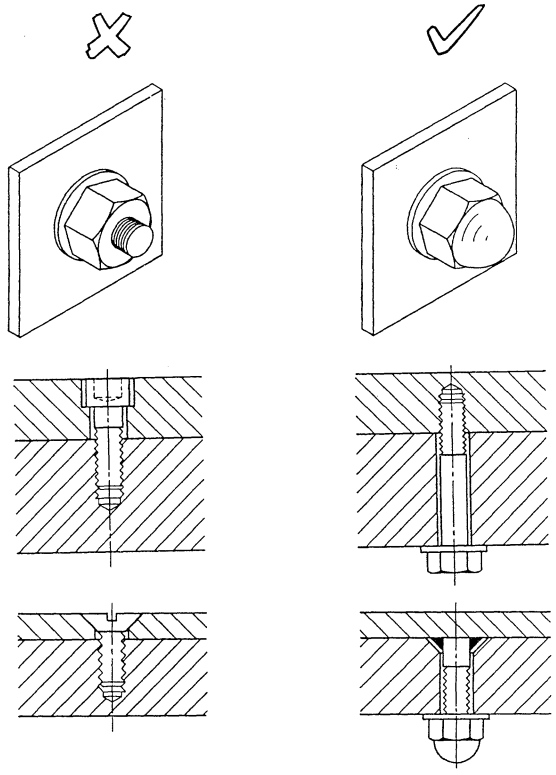


Figure 6. Fasteners

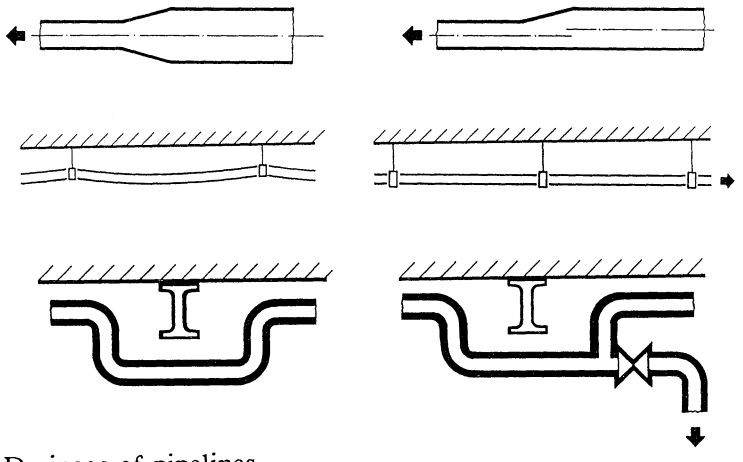


Figure 7. Drainage of pipelines

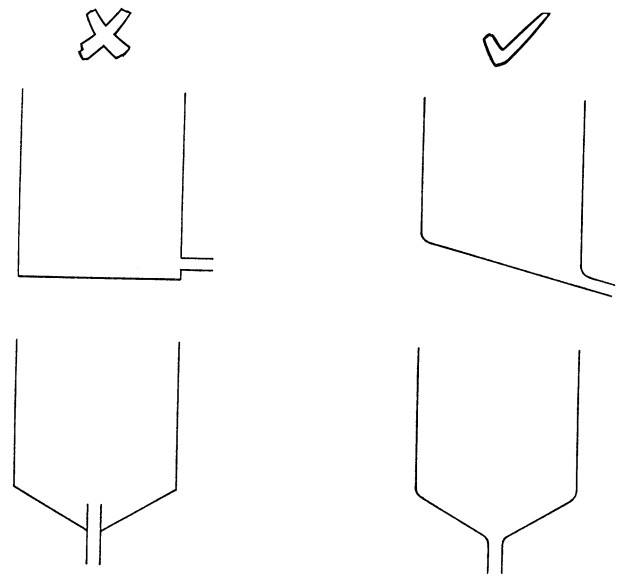


Figure 8. Drainage of vessels

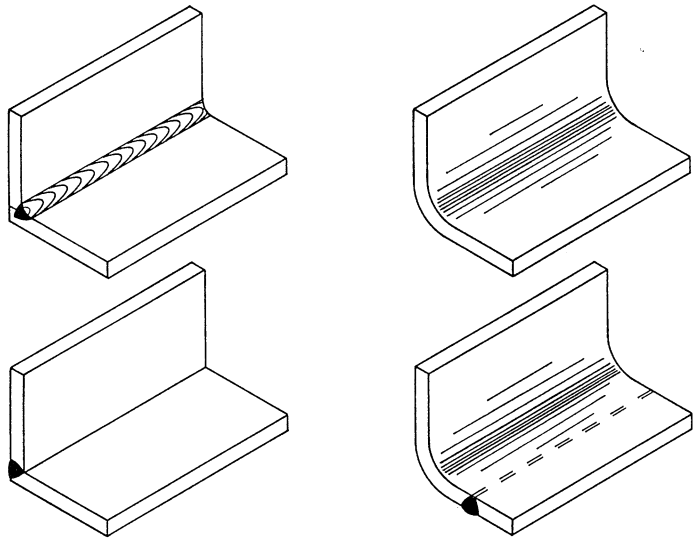


Figure 9. Internal angles

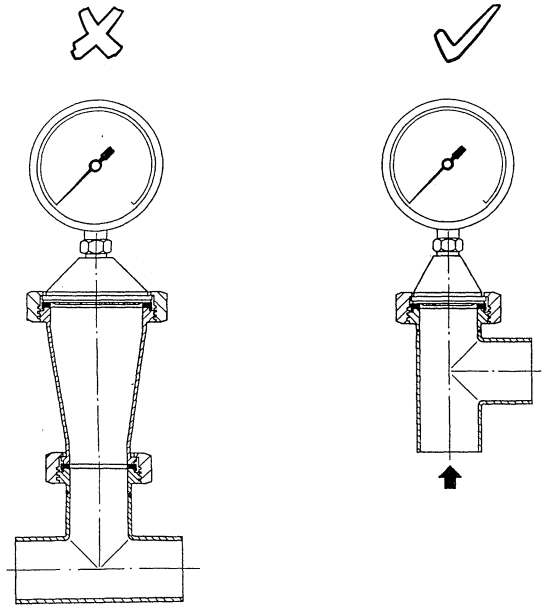


Figure 10. Dead legs

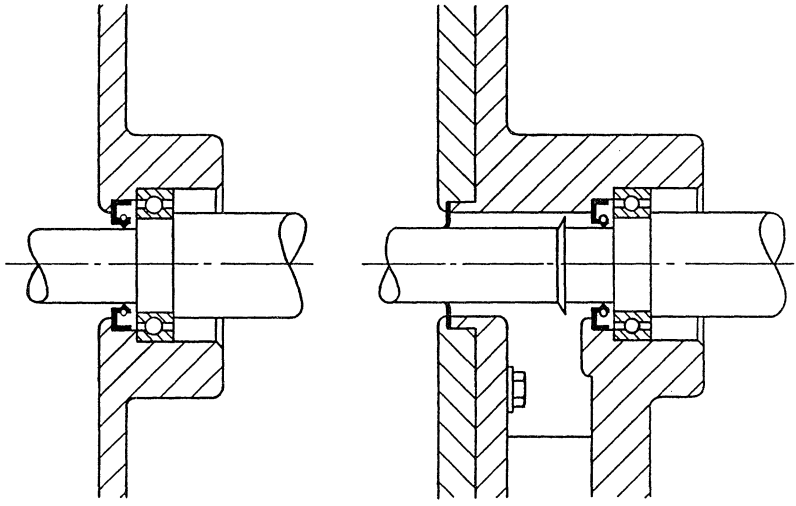


Figure 11. Bearings and shaft seals

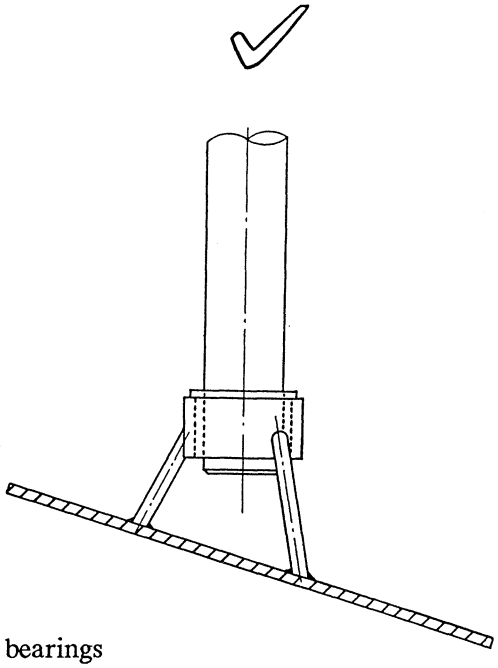


Figure 12. Foot bearings

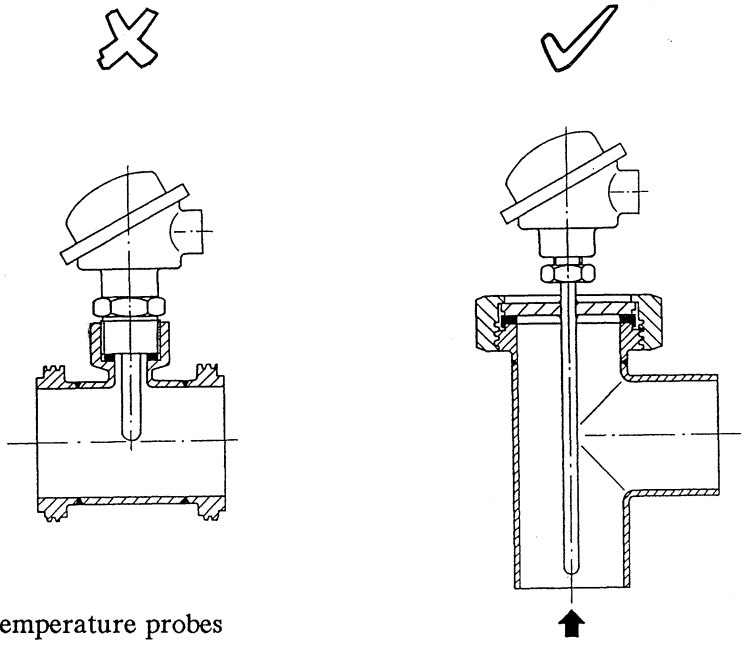


Figure 13. Temperature probes

care must be taken that they are not introduced during installation, Fig. 10, or as the result of modifications.

2.8 BEARINGS AND SHAFT SEALS

Bearings should, wherever possible, be mounted outside the product area to avoid possible contamination of product by lubricants, unless they are edible, or possible failure of the bearings due to the ingress of the product, as shown in Fig. 11. Shaft seals must be of such design as to be easily cleaned and if not product lubricated, then the lubricant must be edible. There are applications where a bearing is within the product area such as a foot bearing for an agitator shaft in a vessel, shown in Fig. 12. In this application it is important that there is a groove completely through the bore of the bush, from top to bottom to permit the passage of cleaning fluid.

2.9 INSTRUMENTATION

Instruments must be constructed from appropriate materials and if they contain a transmitting fluid, such as in a bourdon tube pressure gauge, then the fluid must be approved for food contact. Many instruments themselves are hygienic but often they are installed unhygienically, as shown in Fig. 13.

3. Test Methodology

At the time of writing, the Draft Standard - Basic Concepts - Part 2: Hygiene Requirements (Document N100) states that "open equipment is considered to be cleanable if it complies with the nine basic requirements - and the requirements of specific standards, if any exist". This means that the suitability of open equipment can be established by inspection. Whilst details of verification procedures are still to be published, it is expected that manufacturers of open equipment will be able to self-certify conformance. However, if the equipment is also subject to the Gas or Electrical Directives, there will need to be a more formal procedure, certainly with respect to safety requirements. Closed equipment such as vessels, pipelines, valves and pumps, which are not normally dismantled and are cleaned in place (CIP) will need to be tested. Document N100 states "closed food processing equipment is considered to be cleanable if this can be checked by the means of a standard test of the entire plant or its individual components".

In 1989 an independent group, the European Hygienic Equipment Design Group (EHEDG) was formed, and consists of some 70 members who are research organisations, equipment manufacturers or food processors. The remit of the group is:

- to ensure that food products are processed hygienically and safely
- to provide Standards Organisations with specialist views on hygienic aspects of equipment design
- to ensure that in the future there will be no confusion whether and under which conditions equipment is microbiologically safe for the processing and packaging of food

- to identify areas where knowledge of hygienic and aseptic design, needed to produce recommendations, is insufficient and to encourage research and development in such areas

A Test Methods sub group has been formed to provide hygienic design guidelines that can be verified by test methods and to produce standard test procedures to cover a range of equipment parameters including cleanability, pasteurizability, sterilizability and aseptic capability. Cleanability is of prime importance to all equipment and a method for the assessment of in-place cleanability of food processing equipment is presently being published.

This test procedure is designed to indicate areas of poor hygienic design in equipment where product or microorganisms are protected from the action of the cleaning process and/or to compare pieces of equipment with respect to their in-place cleanability. The method is based on a comparison, in the laboratory, of the cleanability of a test item with that of a straight piece of pipe. The degree of cleanliness is based on the removal of a soil containing bacteria and is assessed by evaluating the number of bacteria remaining after cleaning. The level of cleaning is designed, using a mild detergent, to leave some soil in the reference pipe to facilitate comparisons. The test is intended, therefore, as a basic screening test for equipment hygienic design and is not indicative of performance in industrial cleaning situations.

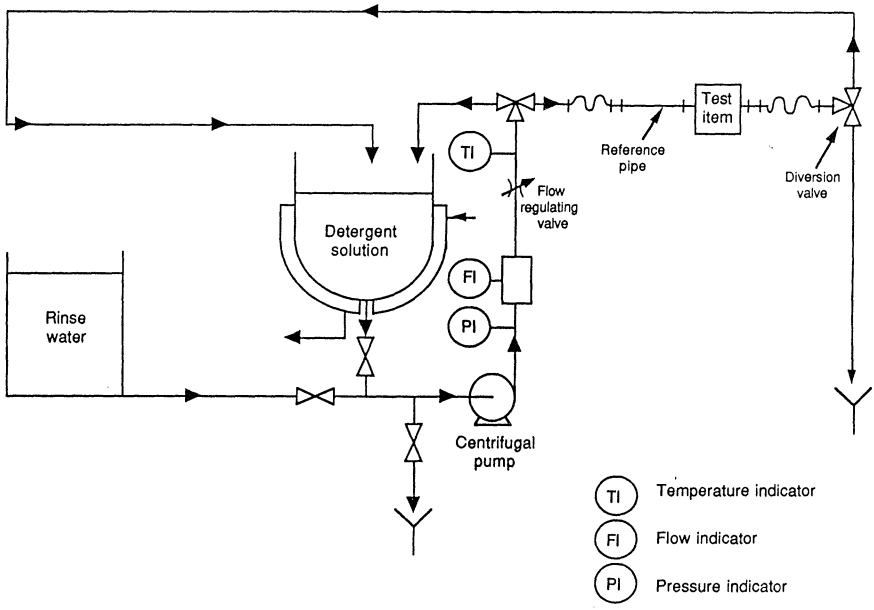


Figure 14. Schematic test rig for the assessment of equipment cleanability

The basis of the test utilises methodology first developed by Galesloot *et al.* (1967) and modified at the Unilever Research Laboratory (Lelieveld 1985) and consists of drying onto the internal surfaces of test items a soured milk soil

containing spores of *Bacillus stearothermophilus*. The test item and reference pipe are then mounted in a purpose built rig (Fig. 14) and cleaned via a cold rinse, a mild detergent clean at 65°C and a final cold rinse using a flow rate of 1.5m/s. The test item and reference pipe are then removed from the rig, dismantled, and all internal surfaces covered with Shapton and Hindes agar. After incubation at 58°C for 24 hours the agar is removed and examined for the presence of yellow areas (from microbial acid production). By comparing the degree of yellow agar and its position and by repeating the test on a number of occasions, a picture can be built up of the relative cleanability of the test item and areas where spores are consistently retained; i.e. areas of poor hygienic design can be identified.

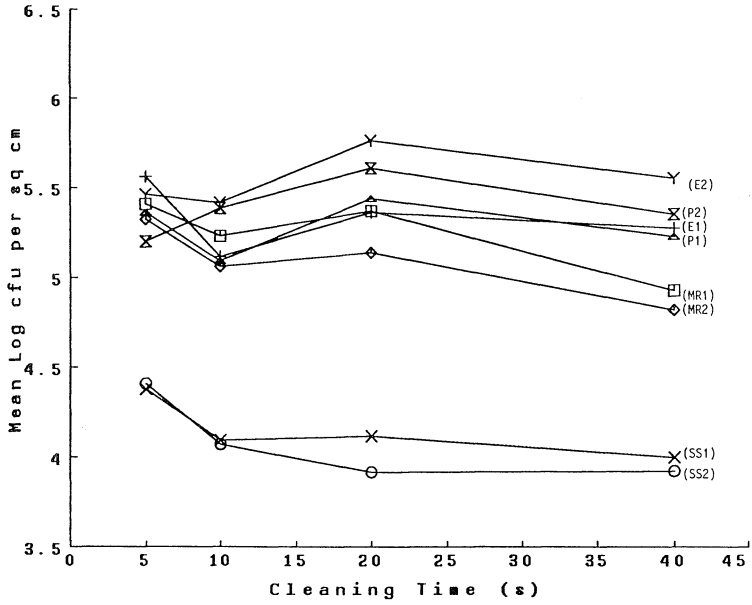


Figure 15. Mean (n=15) log cfu/cm^2 vs cleaning time for duplicate examples of a range of abraded domestic sink materials. SS= stainless steel, E= enamelled steel, MR= mineral resin, P= polycarbonate. (Holah *et al.* 1990)

If this cleanability test method shows good repeatability and reproducibility, it will be recommended to the ad hoc Hygiene Working Group of TC/153 as a possible CEN standard method.

4. Surface Abrasion

The cleanability of materials in relation to material type has been investigated for materials likely to be used in the production or consumption of food. Early work (Ridenour and Armbruster 1953, Masurovsky and Jordan 1960 and Davis 1963) indicated some differences in material cleanability, though later studies using advanced techniques (Holah *et al.* 1988, Holah and Thorpe 1990) showed no significant differences in cleanability between stainless steel, enamelled steel,

mineral resin, high density nylon, PVC, polypropylene and polycarbonate when all materials were in new condition.

When subjected to abrasion or impact damage, however, Holah and Thorpe (1990) demonstrated that stainless steel domestic sink samples retained one log order less bacteria than a number of alternative sink materials (Fig. 15) including enamelled steel, mineral resin and polycarbonate. Further to this, the cleanability of the other materials was not seen to substantially increase with cleaning time. Differential cleanability was correlated with the degree of abrasion/impact damage sustained and assessed by photography and surface roughness measurements. Stainless steel was characterised by smooth shallow grooves, enamelled steel became pitted whilst mineral resin and polycarbonate became extremely rough and jagged with deep pits and crevices.

Results suggested that differential cleanability could be related to surface topography such that the greater the degree of surface change, the greater the retention of microorganisms. This could be due to an increase in bacterial attachment sites (for a given surface area), stronger bacterial adhesion due to increased bacterial/material surface area interfaces and enhanced protection from cleaning sheer forces. Materials that are used in food processing that resist surface changes, typified by stainless steel, will remain more hygienic when subjected to natural wear than materials which become more readily damaged. On abraded or impact damaged materials, bacteria retained after the cleaning regimes applied would, due to the nature of their attachment, be unlikely to be removed with extended cleaning.

References

Anon. (1973) *Metal pipes and fittings- Stainless steel bends and tees for the food industry.* ISO 2851:1973

Anon. (1974) *Metal pipes and fittings- Stainless steel clamp liners with gaskets for the food industry.* ISO 2852:1974

Anon. (1976) *Metal pipes and fittings- Stainless steel screwed couplings for the food industry.* ISO 2853:1976

Anon. (1980) *Metal pipes and fittings- Stainless steel tubes for the food industry.* ISO 2037:1980

Anon. (1982a) *Hygienic design and construction of equipment used in dairy plants.* International Dairy Federation, Bulletin No.153.

Anon. (1982b). The principles of hygienic design for food processing machinery. *Technical Memorandum No. 289.*, CFDRA, Chipping Campden, UK.

Anon. (1983a). The hygienic principles of food processing equipment. *Technical Mannual No.7.*, CFDRA, Chipping Campden, UK.

Anon. (1983b) *Milking machine installations- Construction and performance.* ISO 5707:1983

Anon. (1983c) *Refrigerated bulk milk tanks.* ISO 5708:1983

Anon. (1986) American National Standard ASME/ANSI F2.1-1986

Anon. (1987) *Hygienic design of dairy processing equipment.* International Dairy Federation, Bulletin No.218.

Davis, J.G. (1963) The cleansability of various materials. *The Medical Officer,* 8th November, 301-304

Galesloot, Th.E., Radema, L., Kooy, E.G. and Hup, G. (1967) A sensitive method for the evaluation of cleaning processes, with a special version adapted to the study of the cleaning of tanks. *Netherlands Milk and Dairy Journal* 21, 214-222

Holah, J.T., Betts, R.P. and Thorpe, R.H. (1988) The use of direct epifluorescent microscopy (DEM) and the direct epifluorescent filter technique (DEFT) to assess microbial populations on food contact surfaces. *Journal of Applied Bacteriology* 65, 215-221

Holah, J.T. and Thorpe, R.H. (1990) Cleanability in relation to bacterial retention on unused and abraded domestic sink materials. *Journal of Applied Bacteriology* 69, 599-608

Lelieveld, H.L.M. (1985) Hygienic design and test methods. *Journal of the Society of Dairy Technology* 38, 14-16

Jowitt, R. (1980) *Hygienic Design and Operation of Food Plant.* Ellis Horwood, Chichester, UK.

Masurovsky, E.B. and Jordan, W.K. (1960) Studies on the removal of *Staphylococcus aureus* from milk contact surfaces. *Journal of Dairy Science* 43, 1545

Ridenour, G.M. and Armbruster, E.H. (1953) Bacterial cleanability of various types of eating surface. *American Journal of Public Health* 43, 138-149

Thorpe, R.H. and Barker, P.M. (1985) Hygienic design of post process can handling systems. *Technical Mannual No.8.*, CFDRA, Chipping Campden, UK.

Thorpe, R.H. and Barker, P.M. (1987) Hygienic design of liquid handling equipment for the food industry. Sections 1-3. *Technical Mannual No.17.*, CFDRA, Chipping Campden, UK.

Timperley, D.A., Thorpe, R.H. and Timperley, A.W. (1990) Hygienic design of liquid handling equipment. Sections 4 and 5. *Technical Manual No.17,* CFDRA, Chipping Campden, UK.

Timperley, D.A., Thorpe, R.H. and Timperley, A.W. (1992) Hygienic design of

liquid handling equipment. Section 6. *Technical Manual No.17*, CFDRA, Chipping Campden, UK.

Appendix 1. Machinery groups covered by CEN/TC 153

1. Bakery machines, baking ovens, pasta machines.
1.1 Bakery products, confectionary and pasta equipment.
1.2 Ovens and baking equipment.
2. Machinery and equipment for processing cereals and animal feed.
3. Slaughter-house and butcher's machines and equipment.
3.1 Butcher's machines.
3.2 Slaughter-house machines and equipment.
3.3 Cooking vessels.
3.4 Smoking installations.
4. Fish processing machines.
5. Machines and equipment for fruit and vegetable processing.
5.1 Autoclaves.
6. Machinery and equipment for catering and large kitchens.
6.1 Catering and large kitchen machines.
6.2 Heating/cooking installation.
6.3 Commercial dish washers
7. Machinery and equipment for alcoholic and non-alcoholic drinks.
8. Machinery and equipment for dairies.
9. Whipped cream machines and ice cream machines.
10. Machines and equipment for processing edible oils and fat.
11. Machines and equipment for confectionary and chocolates.
12. Machines and equipment for coffee and tea processing
13. Machinery and plants for the sugar industry.
14. Machines and equipment for tobacco processing.
15. Automatic vending machines.
16. Coolers and freezers.
17. Egg-sorting machinery.

DEVELOPMENT OF MIXED CULTURE BIOFILMS ON STAINLESS STEEL

H.SPENCELEY & C.S.DOW
Biological Sciences,
University of Warwick,
Coventry,CV4 7AL,U.K.

J.T.HOLAH
Campden Food and Drink
Research Association,
Chipping Campden,
Gloucestershire,U.K.

Introduction

Microbial attachment and biofilm formation is ubiquitous having been demonstrated in virtually all environmental niches including surgical implants (Costerton *et al*,1987), stainless steel milk pipelines (Marshall *et al*,1971), and dental caries.

Bacteria attached to a surface have an ecological advantage as being a component of a biofilm offers growth and survival advantages e.g. it has been reported that *Pseudomonas aeruginosa* cells are more resistant to antibiotics and increased resistance in older biofilms was observed (Allison *et al*,1990); attached bacteria have been shown to be more resistant to chlorine due presumably to the production of extracellular polymers(Fletcher,1984). Biofilm bacteria are, therefore, likely to remain on a surface even after cleaning as a potential reservoir of food spoilage and/or pathogenic organisms.

Bacterial attachment is affected by cell hydrophobicity (Van Loosdrecht *et al*,1987b), cell surface charge (Fletcher & Loeb, 1979) and expression of surface structures including exopolysaccharides (Fletcher & Floodgate,1973), fimbriae (Irvin, 1990) and flagella, or any mixture of these components.

This study examines the effects of nutrient limitation and bacterial growth phase on cell surface characteristics such as structural adaptations (polysaccharide production, flagella and fimbriae expression), and hydrophobicity with relation to attachment to stainless steel. Examination of these factors with respect to the individual species from a tri-species biofilm consortium gives an insight into how that community interacts, grows and survives. The consequences for the factory environment are considered in the light of these findings.

This paper presents data for one organism in particular; *Serratia liquefaciens* as part of a consortium which also included *Pseudomonas fragi* and *Staphylococcus cohnii*.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 395–402.

Materials and Methods

1. BACTERIA

1.1 The bacteria used in this study were isolated from a stainless steel surface in a salmon factory, and identified as *Serratia liquefaciens, Pseudomonas fragi* and *Staphylococcus cohnii.*

2. MEDIA AND CULTURE

2.1 Batch cultures were grown at 25°C shaking at 200rpm using the following media. The base was 2g/l K_2PO_4, 1g/l KH_2PO_4, 2g/l Na_2SO_4,and 0.1g/l $MgSO_4$ at pH 7.4. For carbon (C) limitation 1.9g/l NH_4Cl, and 1.9g/l NH_4NO_3 were added and supplemented with filter sterilized glucose (0.1% w/v). For nitrogen (N) limitation 0.1 g/l NH_4Cl, and 0.1g/l NH_4NO_3 were added with 0.5%(w/v) glucose. For C and N excess, additions were as for C limited but with 0.5% (w/v) glucose. The complex media used was Luria-Bertani (LB) which contained 10g/l Bacto-tryptone, 5g/l yeast extract and 10g/l NaCl, pH 7.5.

2.2 Continuous cultures were grown in a 2l chemostat using C and N media as described for batch culture. The dilution rate was 0.022/hr, at 25°C,with an air flow rate of 2.0 l/min and continuous stirring at 500 rpm. The pH was maintained at 7.4.

Stainless steel discs (13mm diameter, type 2B finish, washed in mild detergent and autoclaved) were exposed to the culture in a cylindrical vessel through which the culture fluid was passed at a predetermined flow rate. The discs were held perpendicular to the media flow.

2.3 Attached bacteria were enumerated by immunofluorescent labelling.

3. IMMUNOFLUORESCENT LABELLING

The stainless steel discs were removed from the fermenter system at intervals and washed twice in phosphate buffered saline(PBS), and fixed using 1%(v/v) gluteraldehyde. Indirect labelling used whole cell antisera (raised in this laboratory) and the anti rabbit immunoconjugates used were FITC(Sigma F1262), TRITC(Sigma T5268) and Texas Red(Amersham N2034) as per manufacturers instructions (Harlow & Lane,1988). Attached species were enumerated using a fluorescent microscope and an eye piece graticule. Attachment ratios were calculated to allow for differences in liquid phase cell concentration by dividing cells/cm^2 by cells/ml.

4. BATCH CULTURE ATTACHMENT ASSAY

Aliquots of culture removed through the growth cycle, were washed twice in PBS and resuspended in PBS to give an OD600nm of 0.3 ($3.8x10^8$ cells/ml). Accurate cell concentrations were determined by serial dilution and spread plating onto nutrient agar.

Triplicate sterile stainless steel discs were immersed in washed culture for 1hr at 25°C. The surfaces were removed, washed twice and stained with acridine orange (0.1mg/ml in PBS). The surfaces were washed in PBS and air-dried. Attached bacteria were enumerated by direct epifluorescent microscopy linked to an Optimax image analysis system(Holah *et al*,1988). Attachment ratios were calculated to allow for slight differences in inoculum concentrations.

5. EXOPOLYSACCHARIDE ANAYSIS

Exopolysaccharide was recovered by isopropanol precipitation (Beech *et al*,1991) Total sugars and were assayed using D-glucose as standard (Dubois *et al*,1956), and

uronic acids measured using glucuronic acid as standard (Blumenkrantz & Absoe-Hansen,1973). Results were expressed as μg/g dry wt.

6. HYDROPHOBICITY ASSAYS

Hydrophobicity was measured using bacterial adherence to hexadecane (BATH) (Rosenberg *et al*,1980) and hydrophobic interaction chromatography (HIC) using phenyl-sepharose (Smyth *et al*,1978). Both methods used change in absorbance at 600nm to measure cell retention by the hydrophobic phase.

7. CELL VOLUME ANALYSIS

Coulter Counter Model ZB1 and Coulter Channelizer C1000 connected to a BBC microcomputer were used to determine size distributions.

Results and Discussion

The attachment ratios (Fig.1) show that attachment of *S.liquefaciens* to stainless steel is affected by nutrient limitation and growth phase.

Attachment was found to be maximal during the exponential phase of growth and highest in complex media. There are contradictory reports in the literature as to the effect of growth phase - adhesion has been reported to increase in the exponential phase (Fletcher,1977;Marshall *et al*,1971), whilst others found decreased attachment in this phase (Kjelleberg & Hermansson, 1984).

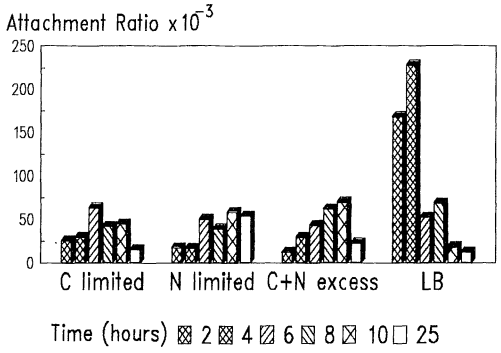


Figure 1 Attachment ratios through the growth cycle of *S liquefaciens*
Stationary phase was reached at the following times C limited 10hrs; N limited 25hrs: C and N excess at 25hrs; LB at 6hrs.

Bacterial adaptations to changes in the environment may be dramatic morphological change eg endospore formation, or structural

adaptations such as production of fimbriae, flagella and polysaccharides, or cell surface changes such as altered hydrophobicity or electrophoretic mobility.

The quantity and composition of lipopolysaccharides, proteins and exopolysaccharides vary with growth condition and growth phase (Ellwood & Tempest,1972). Figure 2 shows that exopolysaccharide production was higher in nitrogen limited cells, with the total sugar results showing the same trends as those for uronic acids. Both *S.liquefaciens* and *P.fragi* synthesized approximately twice as much polysaccharide under nitrogen limitation, whilst *S.cohnii* produces approximately thirty times as much when nitrogen limited. Similar observations are widely reported in the literature (Uhlinger & White,1983).

The fibrous glycocalyx produced by *Pseudomonas putida* and *P.fluorescens* has been shown to be involved in attachment and structural matrices of microcolonies (Moyer,1936). However, since the attachment ratios of carbon and nitrogen limited cells are similar, but exopolysaccharide production is greater in nitrogen limited cells, exopolysaccharide production is probably not an important factor in initial attachment. Evidence that polysaccharides were not involved in initial attachment has been obtained by comparison of wild type attachment with that of a mutant deficient in polysaccharide synthesis, and that polymer production is necessary for microcolony formation (Allison & Sutherland,1987). Our data on polysaccharide production by attached bacteria has shown that production is between two and five times greater than liquid phase cells depending on the organism.

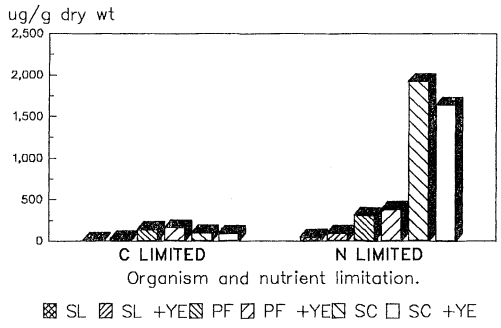


Figure 2 Comparison of exopolysaccharide production by carbon and nitrogen limited cells as measured by total sugar.
SL *S.liquefaciens*, PF *P.fragi*, SC *S.cohnii*. YE; 0.01% dialysed yeast extract added.

Hydrophobicity was shown to be dependent upon growth conditions (Fig.3). Both *S.cohnii* and *P.fragi* showed increased hydrophobicities in limited media. Hydrophobic organisms have been shown to adhere to a greater extent than hydrophilic ones (Van Loosdecht *et al*,1987b) and

that in a limiting environment these organisms would have an ecological advantage as they would be more likely to attach to a solid liquid interface where there is accumulation of nutrients(Fletcher & Marshall,1982). In contrast *S.liquefaciens* became slightly more hydrophobic in limited media. These trends were also observed when hydrophobicity was measured by HIC , although the hydrophobicity values were higher. HIC measures hydrophobic interaction between the bacterial cells and non polar phenyl ligands, whilst BATH measures adhesion to hydrocarbon where interfacial tensions between the water and the organic phase are high (Kjelleberg,1984). HIC is the more sensitive method for hydrophilic organisms, whilst BATH is thought to be more appropriate for hydrophobic organisms(Gilbert *et al*,1991).

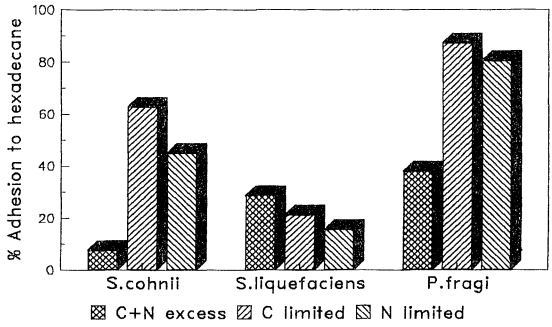


Figure 3 Comparison of cell hydrophobicity in different media measured by BATH.

Measurements of the hydrophobicity of *S.liquefaciens* through the growth curve showed that the hydrophobicity decreased in the exponential phase for a short time and then increased again. A similar change in hydrophobicity has been demonstated in *Escherichia coli* and *Staphylococcus epidermidis* although the period of reduced hydrophobicity was longer, but this may be due to slower growth curves compared to *S.liquefaciens* (Gilbert *et al*,1991). This reduction in hydrophobicity is thought to be a cell cycle effect as daughter cells released from a biofilm were more hydrophilic than the biofilm or planktonic cells (Allison *et al*,1990). They showed that increasing the growth rate reduced the hydrophobicities of biofilm and planktonic cells only, daughter cells remained consistently hydrophilic.

Cell volume analysis through the growth curve showed an increase in mean cell volume during the exponential phase, when cell division is maximal, and decreased towards stationary phase. If daughter cells are more hydrophilic then hydrohobicity would be at its lowest value when mean cell volume, and therefore cell division, are maximal (Fig. 4)

There is a correlation between attachment of *S.liquefaciens* and mean cell volume; attachment is at its maximum when mean cell volume is at its maximum value. This confirms the existence of a

heterogeneous population in that the hydrophilic daughter cells are unlikely to attach, whilst older cells with increased surface area and higher hydrophobicities are more likely to attach.

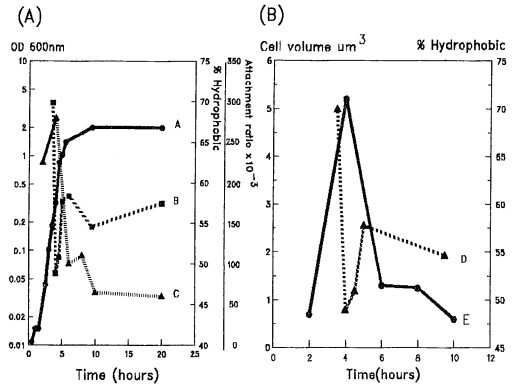


Figure 4 Comparison of (A) hydrohobicity and attachment and (B) hydrophobicity and cell volume through growth of *S.liquefaciens* in LB.
A OD600nm B Hydrophobicity(HIC) C Attachment ratio
D Hydrophobicity(HIC) E Mean cell volume

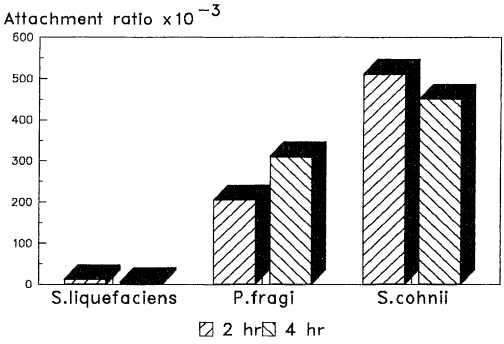


Figure 5 Comparison of attachment ratios from a C limited mixed culture fermenter. Surfaces were removed after two and four hours and attached bacteria enumerated by immunofluorescent labelling.

In contrast to other workers who found that high hydrophobicity correlated with higher levels of adhesion (Van Loosdrecht *et al*,1990), we found that whilst hydrophobicity decreased during the exponential phase ,this was when attachment was maximal. The adhesion of hydrophobic organisms is dominated by thermodynamic features of the cell surface such as surface hydrophobicity, irrespective of surface

charge. Electrokinetic potential plays a more important role in the adhesion of relatively hydrophilic organisms (Van Loosdecht *et al*,1987b). *S.liquefaciens* is a relatively hydrophilic organism and this may explain the lack of correlation between attachment and hydrophobicity.

The attachment ratios of *S.liquefaciens* from mixed culture are approximately three times less than those from single species in batch culture (Fig. 5).

Although *S.liquefaciens* is the dominant organism in the liquid phase , because of the various components involved that dictate its attachment ability , it attaches relatively poorly compared to the other two species. *P.fragi* and *S.cohnii* are highly hydophobic organisms and tend to attach more readily. This factor alone may explain their high attachments ratios , but their attachments abilites are more likely to be the net result of various factors.

Acknowledgement

Financial support from MAFF and Campden Food and Drink Research Association is gratefully acknowledged

References

Allison, D.G. & Sutherland, I.W. (1987) The role of exopolysaccharides in adhesion of freshwater bacteria. J. Gen. Microbiol. 133, 1319-1327.

Allison, D.G.,Brown, M.R.W.,Evans, D.E. & Gilbert, P. (1990) Surface hydrophobicity and dispersal of *Pseudomonas aeruginosa* from biofilms.FEMS Micro. Lett. 71,101-104.

Beech, I.B., Gaylarde, C.C., Smith, J.J. & Geesey, G.G. (1991) .Extracellular polysaccharide from *Desulfovibrio desulfuricans* and *Pseudomonas fluorescens* in the presence of mild and stainless steel. Appl. Microbiol. Biotechnol. 35, 65-71.

Blumenkrantz, N. & Absoe-Hansen, G. (1973).New method for quantitatine determination of uronic acids Analytical Biochem. 54, 484-489.

Costerton, J.W., Cheng, K.J., Geesey, G.G.,Ladd, T.I., Nickel, J.C., Dasgupta, M. & Marrie,T.J. (1987)Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. 41, 435-464.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F. (1956) Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28,350-356.

Ellwood, D.E., & Tempest, D.W. (1972)Effects on the environment on bacterial cell wall content and composition. Advances in Microbiol Physiology 7, 83-117.

Fletcher, M. (1977)The effects of culture concentration and age, time and temperature on bacterial attachment to polystyrene. Can. J. Microbiol. 23, 1-6.

Fletcher, M. (1984). In Microbial Adhesion & Aggregation. Ed. Marshall, K.C. Springer-Verlag.

Fletcher, M., & Floodgate, G.D. (1973) An electron microscope demonstration of an acid polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. J. Gen Micrbiol. 74, 325-334.

Fletcher, M. & Loeb, G.I. (1979).The influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces Appl. Environ. Microbiol. 37, 67-72.

Fletcher, M. & Marshall, K.C. (1982) Bubble contact angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment. Appl. Environ. Microbiol. 44, 184-192.

Gilbert, P., Evans, E., Duguid, I.G. & Brown, M.R.W. (1991) Surface characteristics and adhesion of *Escherichia coli* and *Staphylococcus epidermidis* J. Appl.Bacteriol. 71, 72-77.

Harlow, E. & Lane, D. (1988). In Antibodies;A Laboratory Manual. Chapter 10. Cold Spring Harbour Laboratory.

Holah, J.T., Betts, R.P. & Thorpe, R.H. (1988) The use of direct epifluorescent microscopy (DEM) and the direct epifluorescent technique (DEFT) to assess microbial populations on food contact surfaces. J. Appl. Bacteriol. 65, 215-221.

Irvin, R.T. (1990). In Microbial Cell Hydrophobicity, Ed. Doyle, R.J. & Rosenberg, M. American Society for Microbiology, Washington DC.

Kjelleberg, S. (1984). In Microbial Adhesion and Aggregation, Ed. Marshall, K.C. pp. 51-70. Berlin;Springer Verlag.

Kjelleberg, S. & Hermansson, M. (1984) Starvation induced effects on bacterial surface characteristics. J. Appl. Environ. Microbiol. 48, 497-503.

Marshall, K.C., Stout, R. & Mitchell, R. (1971) Mechanism of the initial events in the sorption of marine bacteria to surfaces. J. Gen Microbiol. 68, 337-348.

Moyer, J.R. (1936) Changes in electrokinetic potential of bacteria at various phases of the culture cycle. J. Bacteriol. 32,433-464.

Rosenberg, M. Gutnick,D. & Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9, 29-33.

Smyth, C.J., Jonsson,P., Olosson, E., Soderlind,O., Rosengren,J., Hjerten, S. & Wadstrom, T. (1978) Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with and without K88 antigen as revealed by hydrophobic interaction chromatography Infect. Immun. 22, 462-472.

Uhlinger, D.J. & White, D.C. (1983) Relationship between physiological status and formation of extracellular polysaccharide glycocalyx in *Pseudomonas atlantica.* Appl. Environ. Microbiol. 45, 64-70.

Vanhaecke, E.,Remon, J-P., Moors, M., Raes, F., Rudder,D. Van Peteghem, A. (1990) Kinetics of Pseudomonas aeruginosa adhesion to 304 to 316-7 stainless steel ; role of cell surface hydrophobicity Appl.Environ. Microbiol. 56, 788-795.

Van Loosdrecht, M.C.M, Lyclema, J., Norde, W., Schraa, G. & Zehnder, A.J.B. (1987b) Electrophoretic moboility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion. Appl.Environ. Microbiol. 53, 1898-1901.

Van Loosdrecht, M.C.M, Lyclema, J., Norde, W., Schraa, G. & Zehnder, A.J.B. (1990)Influences of interfaces on microbial activity.Microbiol. Rev. 54, 75-87.

ADHESION OF *Bacillus cereus* SPORES - A HAZARD TO THE DAIRY INDUSTRY

U. RÖNNER / U. HUSMARK
SIK, The Swedish Institute for Food Research
P.O. Box 5401
S-402 29 GÖTEBORG
Sweden

1. Introduction

The dairy industry has problem with infections of *B. cereus*, especially during August - October every year (Donovan, 1959; Phillips & Griffiths, 1986; Sogaard & Petersen, 1977 and Walkers *et al.*, 1989). High levels of *B. cereus* lead to difficulties for the dairy industry to produce milk of "approved" quality.

In order to fulfil the legal requirements (< 10^4/ml after 6 days at + 8°C) for milk, no more than 10 spores/100 ml are allowed at the packaging line. Therefore, it is very important to trace the presence of spores from farmer to package, in order to determine if poor procedures on the farms or if an ineffective cleaning process in the dairy plant is the weak point.

To increase understanding of infection problems, an investigation of the presence of *B. cereus* spores in a dairy plant was conducted. If *B. cereus* spores are present, the study will aim on questions such as: does *B. cereus* have features which make it better fit than other present microorganisms, suggesting that ahesion potential may be such a factor, since it has been reported that *B. cereus* spores are very hydrophobic and also very adhesive to different solid surface materials (Ronner et al., 1990)?

2. Methods

2.1 EVALUATING THE NUMBER OF SPORES IN MILK

The sampling points of a dairy plant are marked with a star on the illustration below.

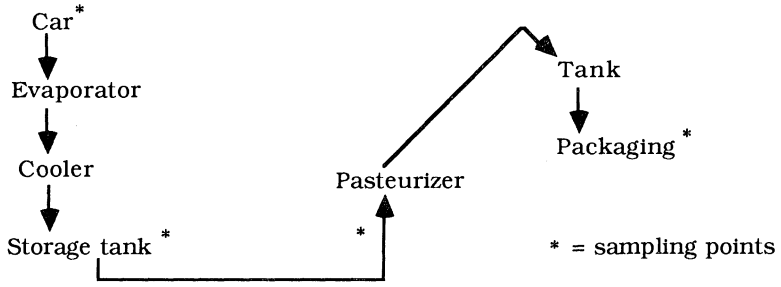


Figure 1 - Process line of milk.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 403–406.

The samples (100 ml) were filtrated (0.8 um) and treated according to Abgrall *et al.* (1987). Basically the filter was put on a blood agar plated and incubated. Developed colonies were counted and *B. cereus* colonies were further confirmed on Mossel agar (Merck, 5267).

2.2 EVALUATING THE NUMBER OF SPORES AFTER CLEANING

At the sampling point before the pasteurization (see illustration) stainless steel plates were inserted into the pipeline on the raw milk side before the pasteurizer. These plates were fouled with milk and infected by laboratory produced *Bacillus cereus* spores (NCTC 2599). Production procedures for the spores have been described in Husmark & Ronner (1992). The infected stainless steel plates were thereafter exposed to a regular cleaning programme. The cleaning programme sequence was as follows:

Process	Temp.	Time	
Cold water	10°C	90 sec	
↓			
Rinse water	20°C		
↓			
Alkali 1%	75°C	420 sec	NaOH
↓			
Rinse water	20°C		
↓			
Acid 0.8%	70°C	240 sec	HCl
↓			
Cold water	10°C	130 sec	

Figure 2 - Cleaning program.

After the cleaning process the stainless steel plates were removed from the process plant and examined.

The procedure started with a regular swab-technique where the remaining spores were transferred into tubes of saline solution (0.85%). After filtration of the saline solution (0.85%) the filter was treated in the same way as described for the milk samples.

3. Results

The monitoring of *B. cereus* spores in a dairy plant during the monts of September - October along a process line previously described resulted in a decreased level from tanker to packaging.

TABLE 1: *B. cereus* spores/100 ml of milk at four sampling points in the dairy.

Tanker $\bar{x} \pm SD$	Storage $\bar{x} \pm SD$	At pasteurizer $\bar{x} \pm SD$	Packaging $\bar{x} \pm SD$
26.4 ± 15.6	11.1 ± 7.2	7.7. ± 4.8	3.7 ± 1.6
n = 8 (number of samples)			

Evaluation of the cleaning process showed that there are many *B. cereus* spores surviving and still attached to the stainless steel surfaces after cleaning.

The results are given in Table 2.

TABLE 2: Number of *B.cereus* spores on stainless steel surface before and after the cleaning process.

	Before cleaning $\bar{x} \pm SD$	After cleaning $\bar{x} \pm SD$	% reduction
Number of spores / cm^2 mean	75 ± 33	44 ± 21	41
n = 4 (number of plates)			

4. Discussion

In order to explain the decreasing level of *B. cereus* spores along the process line (Table 1), two scenarios are possible. The first theory is that germination of spores occurs along the process line. Milk is a good nutrient but the temperature is generally too low and time too short for spore outgrowth to seem likely (Strange & Hunter, 1969).

A more probable explanation to the results presented in Table 1 is the adhesion of *B. cereus* spores to the equipment surfaces along the production line. *B. cereus* spores have been shown to be very adhesive to different solid surfaces. The spore state is much more adhesive than the vegetative state and *B. cereus* spores are the most adhesive of different Bacillus species tested (Ronner *et al.* , 1990). The following three features of *B. cereus* spores — its surface has a high relative hydrophobicity, low zeta-potential and morphologic features, such as long appendages — all promote a successful adhesion to various solid surfaces (Husmark and Ronner, 1992).

From the data in Table 1 a scenario could be suggested as to how *B. cereus* cause infection in a dairy plant: Spores enter the dairy plant via the milk transport system and some of them will then adhere to the inner surfaces of the milk pipeline. At the point of pasteur the vegetative cells will be killed off, but not the spores which have a good heat resistance. However, the pasteurizer may activate the spores, which can

lead to germination and eventually outgrowth to vegetative cells. As vegetative cells, a great deal of their adhesive capacity will be lost (Ronner *et al.*, 1990). They may detach, enter the milk flow and finally enter the packaging section. If this occurs in too large numbers the shelf life of that milk package will be reduced to an unacceptable level.

In order to reduce the level of spores present in the process lines, cleaning procedures are conducted. The results suggest that the cleaning of the surface fouled with milk and with adhered spores was not very effective. The cleaning process removed or killed only 41% of the attached population. The spores tenaciously adhere to the surface after the initital adhesion and apparently they are very difficult to detach with current cleaning procedures, which do not provide strong enough shear forces for the removal of spores.

REFERENCES

Abgrall, B., Colin, J.Y. and Bourgeois, C. (1987) "Mise au point d'une methode et d'un appareil semi-automatique de filtration sous pression pour le denombrement des spores de *Clostridium tyrobutyricum* dans le lait". Sci. Aliments 7: 157-164.

Donovan, K.O. (1959) "The occurance of *Bacillus cereus* in milk and on dairy equipment". J. of Applied Bacteriology 22: 131-137.

Husmark, U. and Ronner, U. (1992) "The influence of hydrophobic, electrostatic and morphologic properties on the adhesion of baccillus spores". Biofouling 5: 335-344.

Phillips, J.D. and Griffiths, M.W. (1986) "Factors contributing to the seasonal variation of *Bacillus spp.* in pasteurized dairy products". J. of Applied Bacteriology 61: 275-285.

Ronner, U., Husmark, U. and Henriksson, A. (1990) "Adhesion of bacillus spores in relation to hydrophobicity". J. of Applied Bacteriology 69: 550-556.

Sogaard, H. and Petersen, E. (1977) "The occurence of *Bacillus cereus* in dairy products". Nordisk Veterinar Medicin 29: 429-435.

Strange, R.E. and Hunter, J.R. (1969) "Outgrowth and the Synthesis of Macromolecules", in Gould and Hurst (eds). The bacterial spore, Academic Press, pp. 445-480.

Walker, S.J., Archer, P.A. and Banks, J.G. (1989) "Growth of Pathogenic Bacteria at Chill Temperatures. II. *Bacillus cereus*". Campden Food & Drink Research Association Technical Memorandum Nº 535 MAFF Nº 600.

Chapter 7

BIOFILMS IN WASTEWATER TREATMENT AND BIODEGRADATION OF WASTES

BIOFILM REACTORS TECHNOLOGY IN WASTEWATER TREATMENT

R. MENDEZ and J.M. LEMA
Department of Chemical Engineering
University of Santiago de Compostela
Av. de las Ciencias s/n
E-15706 Santiago de Compostela (Spain)

1. Introduction

The observation of the mechanisms used by nature to do a self-depollution of wastes, allowed to develop different systems involving different organisms. There are many living organisms capable to carry out different biochemical reactions that can be exploited in wastewater treatment.

The main problem to use these organisms is to design a system, with the appropriate environmental conditions, that can maintain a significant quantity of different microorganisms capable to degrade the organic material contained in sewage and industrial effluents.

The first systems constituted by Trickling Filters and Activated Sludge Units were designed and operated efficiently during the first decades of the present century. In the fifties and sixties aerobic processes were in vogue for biological treatment of wastewater - even to the extent that anaerobic digestion of sludges were exchanged with other processes for sludge stabilization. The picture changed significantly as a result of the environmental debate and increase in energy prices. Reuse and energy conservation became the words of the day and anaerobic processes quickly emerged with a new potential. It is interesting to note that the basis of anaerobic processes has been studied before and that much information already existed regarding the traditional approaches.

The main problems of the traditional aerobic systems are the high consumption of aeration energy and the enormous quantities of sludge produced that it is necessary to stabilize before to be dumped. On the contrary the anaerobic processes are in practice exceedentary of energy (biogas) and produce a considerably lower quantity of stabilized sludge.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 409–419.

Although initially it was established a certain competition between aerobic and anaerobic processes, now it seems that the application of both technologies would be complementary; so it is everyday more frequent to see wastewater treatment plants where an anaerobic treatment is used in a first step to remove most of the organic material and an aerobic process is used in a further step to treat the residual organic material coming out of anaerobic digester. This complementarity is now being exploited to design systems allowing to remove not only the carbonaceous material but also the nutrients like nitrogen and phosphorus, present in most of wastewaters containing organic material.

2. Technologies

There are two mechanisms allowing to maintain a high concentration of active biomass into the biological reactors: i) By using some kind of support that retains the biomass by the development of biolayers, or ii) By promoting the formation of bacterial aggregates with high setteable properties, sometimes complemented with the recycling of the biomass from an external clarifier.

These mechanisms were initially developed in aerobic conditions, being the trickling filter and the activated sludge process examples of both types of systems. These processes have success in the treatment of medium- and low-loaded wastewaters and they are being extensively used in the treatment of sewage.

The aerobic processes present several operational problems, such as the high requirement of energy to aerate the wastewater and the high production of sludges as a result of aerobic bacteria metabolism. Otherwise, they fail when high-loaded wastewaters are introduced into the system.

A considerable research was carried out during the last two decades to develop efficient systems able to work with anaerobic bacteria to avoid the consumption of energy, and also the high productions of sludges. Nowadays there are a significant number of anaerobic digesters working properly all over the world, treating all kinds of high organic-loaded industrial wastewaters. Similarly to the aerobic technology two basic types of digesters were developed: Attached and non attached biomass reactors.

A classification of the different kinds of aerobic and anaerobic systems used in the wastewater treatment is presented in Table 1.

2.1. AEROBIC PROCESSES

The aerobic reactors where microorganisms are retained mainly by attaching are:

The Trickling Filter that is a system where the wastewater circulates downflow in countercurrent with upflow air through a colonized packing material. The Submerged Filter is a variant where biofilm is completely submerged into the wastewater and the air is introduced at the bottom of biofilter.

The Rotating Biological Contactor (RBC), also named Rotating Biodiscs consists of a series of discs colonized by aerobic organisms that are alternatively in contact with wastewater and air.

TABLE 1. Classification of wastewater treatment bioreactors

Type of Reactor	Aerobic	Anaerobic
Attached biomass		
Fixed Bed	Trickling filter	AF
	Submerged filter	DSFFR
Moving Bed	RBC	ARBC
Expanded Bed	AeEBR	EBR
Fluidized Bed	AeFBR	FBR
Recycled Bed	AeRB	CASBR
Non-attached biomass		
Recycled flocs	Activated Sludge	Contact Process
Sludge blanket	--	UASB

In the Aerobic Expanded (AeEBR), and Fluidized Bed Reactors (AeFBR) the microorganisms are attached to inert support particles, maintained in suspension or in fluidized conditions. Wastewater and air enter into the reactor for the lower part, and an important recycling flow is used to maintain the operational conditions.

The Aerobic Recycled Bed (AeRB) uses also inert support materials to retain the biomass, but there are an important fraction of biomass in suspension; so an external device (sedimentation tank) is used to retain and recycle the biomass that come out of the reactor.

The aerobic reactors that work with non-attached microorganisms are the Activated Sludge units, commonly used in the sewage treatment. In these units the microorganisms are suspended with wastewater and air in the mixed liquor. A secondary clarifier is used to separate the sludge coming out of the unit that is recycled in part to maintain an appropriate concentration of biomass into the reactor.

There are also many full-scale installations where the different aerobic technologies are used jointly in order to get the better results, taking the best advantages of each one.

2.1.1 *Trickling Filter.* These equipments, also named "percolation beds" are one of the first systems used in the wastewater treatment. For a long period of time Trickling Filters were displaced by Activated Sludge units, because these units provide a higher level of treatment and certainly provide greater operational flexibility. However, in the eighties the trickling filter has re-emerged in several new applications (Parker *et al.* 1990) based in its low energy requirements and operating costs. These applications include both the elimination of carbonaceous material -by using together with an aerated solids contact and a flocculator- and its use as nitrification unit (Biofilm controlled nitrifying trickling filter).

The biofilms at TF present a high stratification both along the filter (higher substrate concentrations and lower availability of oxygen at the top than at the bottom section) and at flow transversal section (high availability of substrate and oxygen on the biofilm surface, and low availability of substrate and anaerobic conditions on the biofilm-support contact surface). By other hand, TF can be operated at high- and low-loaded conditions, so it is very difficult to establish a good model for the performance of the filter.

Because of the complex interrelationships between process variables, no good mechanistic model that will accurately predict filter performance has been developed, being also used for design purposes different empirical or semi-empirical equations. Some of the most popular design equations are the National Research Council (NCR), the Galler-Gotaas or the Eckenfelder equations (Benefield and Randall, 1987). Recently there are a considerable interest in studying on scientific and engineering basis the behaviour of TF, to know how hydrodynamics, type of materials and other factors are related with the microbial processes (Crine, *et al*, 1990). The use of submerged biofilters is also being investigated.

Experimental results (La Motta, 1976) have proved that the substrate removal increases linearly as the biofilm thickness increases, until to reach the maximum substrate removal rate that corresponds to a critical size of the biofilm depending on the operational conditions.

Although the thickness of biofilm can be relatively important (till 5-8 mm), the higher COD removal from the effluent is centered basically in a very thin (0.06-0.2 mm) superficial active layer working in aerobic conditions. The sizes of biofilm and active layer depend not only on applied organic load or oxygen availability, but also on wastewater nutrients content and metabolic activity of organisms in the biofilm. It is reported (Harris and Hasford, 1976) that an oxygen limitation can be observed when the organic concentration of substrate is higher than 400 $gCOD/m^3$.
Some results of high rate TF are presented in Table 2 (Eckenfelder, 1989).

TABLE 2. High-rate trickling filter performance

Waste	HLR (m^3/m^2d)	Depth (m)	BOD (mgO_2/L)	Recycle ratio	BOD_r (%)	OLR ($gO_2/L.d$)
Sewage	60-235	3-6.5	145-185	0-3	45-88	0.8-9
Citrus	67-176	6.5	464-542	2-3	42-69	3-10
Kraft mill	84-340	5.5-7	250	-	33-40	-
Black liquor	44-147	5.5	400	-	24-35	3-12

2.1.2 *Rotating Biological Contactors*. This technology is being extensively studied, not only because of its capacity for COD removal, but also for its capacity to remove nitrogen by nitrification/ denitrification.

There are many authors studying the mechanisms of COD removal (Bovendeur, *et al.*, 1990), defining the kinetics and new mathematical models (Capdeville and Nguyen, 1990; Gujer and Boller, 1990), or evaluating the influence of discs material on the performance of the equipments (Ware, *et al.*, 1990).

Considerable efforts are being made in the study of nitrification/denitrification capacity of RBC. Biofilm properties (Masuda *et al.* 1990), and different operational conditions are being reviewed. These equipments are being used in the treatment of industrial effluents, having high capacity to remove not only COD or SS, but also different products like sulphide or Cr^{3+} (Yang and Fan, 1990).

2.1.3 *Fluidized Bed Reactors.* This aerobic system is conceptually very similar to the Anaerobic Fluidized Bed Reactors that will be analyzed later with more detail.

Literature reports a certain number of papers studying different advanced aspects like the hydrodynamics and biomass distribution (Hermanowicz and Cheng, 1990), nitrification (Cooper and Williams, 1990), denitrification (MacDonald, 1990), or specific problems like the biological oxidation of ferrous iron in high acid mine drainage (Omura, *et al.* 1991). There are also reviews where the state of the art is studied in detail (Sutton and Mishra, 1990).

The efficiency of this system is limited by the aeration, as it is very difficult to supply air at such as rate that allow to maintain significant levels of oxygen in the equipment.

2.2. ANAEROBIC PROCESSES

The Anaerobic Filter Reactor (AF), also refered as packed-bed reactors, is provided for microbial attachment with an inert support material arranged in sheet, ring or sphere conformation and packed in loose-fill (random packed) or ordered (modular) configuration. The reactor may operate in upflow or downflow feed mode, with recycling, and usually presents in practice an important retention of occluded biomass.

The Downflow Stationary Fixed Film Reactor (DSFFR) is a variant of the AF, but in this design, the oriented support material provides a surface for biofilm formation and leaves open channels for gas release and suspended solids settlement.

The Anaerobic Rotating Biological Contractor (ARBC) is an adaptation of the aerobic RBC, but until now it is used only at lab scale.

The Anaerobic Expanded Bed (EBR) and the Fluidized Bed Reactors (FBR) use fine carrier particles to retain the active biomass by attachment to the carrier surfaces. The particles, having the attached biofilm, are fluidized by high upflow liquid velocities, generally produced by a combination of the influent and recycling flows. The rate of liquid flow and the consequent degree of expansion of the bed determines whether the reactor is termed as fluidized-bed or expanded-bed reactor.

The Carrier Assisted Sludge Bed reactor (CASBR) (Martenson and Frostell, 1982), is not a very common system, that uses also inert support materials to retain the biomass, but there are an important fraction of biomass in suspension. The bed is kept suspended by mechanical stirring and gas stirring, so an external sedimentation tank is used to recycle the attached biofilm and suspended settled flocs that come out of the reactor.

The Upflow Anaerobic Sludge Blanket (or Bed) Reactor (UASB), developed in the Netherlands, (Lettinga *et al.*, 1980), relies on the tendency of anaerobic bacteria to form flocs or granules which are retained within the reactor by an efficient gas/solid/liquid separation device located at the top of the reactor. With some

TABLE 3. Technical data, anaerobic treatment of wastewater with fixed film reactors.

	Fixed bed	Moving bed	Expanded bed	Fluidized bed	Recycled bed
Inert material, type	Gravel/ plast	Plast	Sand/ Gravel/ Plast	Garnet/ Sand/ Carbon	Sand
Inert material, diameter (mm)	20-50	1000-3000	0.3-3	0.2-1	0.01-0.1
Inert material, disk spacing (cm)	-	10-20	-	-	-
Inert material, rotation (rpm)	-	2-5	-	-	-
Inert material, peripheral velocity (m/s)	-	0.3	-	-	-
Inert material, submergence (%)	100	75-100	100	100	100
Porosity, empty bed (%)	40-98	-	-	-	-
Porosity, operation (%)	20-90	-	-	-	-
Bed expansion (%)	-	-	20-40	30-100	-
Specific surface area (m^2/m^3)	60-200	100-200	1000-3000	1000-2500	2000-5000
Height of reactor (m)	3-6	-	2-4	4-8	5-10
Radius of reactor (m)	(5-20)	1-3	2-3	2-3	(5-20)
Vertical velocity, empty bed (incl. recycle) (m/h)	0.01-0.10	-	2-10	6-20	-
Recycle ratio	-	-	2-100	5-500	0.5-2
Biomass concentration (kg SS/m^3)	5-15	5-15	10-30	10-20	5-15
Attached biomass (% of total)	20-80* 50-90**	50-80	90-100	95-100	0
Suspended biomass (% of total)	20-80* 10-50**	20-50	0-10	0-5	100
Suspended solids, effluent (g SS/m^3)	20-300	20-300	20-100	20-100	20-100
Gasflux, vertical (Nm^3/m^2 d)	5-20	-	5-40	5-40	-
Gasflux, vertical maximal (Nm^3/m^2 d)	10-20	-	30-40	30-40	-
Energy, pumping (Wh/m^3)	-	-	10-20	15-30	-
Energy, pumping, (incl. recycle) (Wh/m^3)	20-40	5-10	20-1000	75-3000	10-30
Energy, rotation (Wh/m^3 tank)	-	20-80	-	-	-
Energy, mixing (Wh/m^3 tank)	-	-	-	-	5-15

* upflow
** downflow

wastewaters, the retained biomass develops in a highly granular sludge with excellent settling properties and forms a dense sludge within the reactor. However, a good performance can be also obtained with non-granular (flocculent) sludge.

In the Contact Process, also referred as the anaerobic activated sludge process, the suspended biomass is separated in an external gravity or centrifugal separating device and returned to the reactor. A proper degasification is necessary if settlement is applied.

A summary of the main characteristics of the different anaerobic bioreactors working with important fractions of attached biomass (Hence and Harremoes, 1983) is presented in table 3.

During the last years some of the reactors presented in table 3 became more important than others, and appeared new variants. The Hybrid reactors consist of different combinations among the above basic types. For example, limitation of the support matrix to the upper section of the reactor gives a hybrid AF in which the unpacked section functions like an UASB reactor; the Anaerobic Baffled Reactor (ABR) provides a combination of several UASB reactors in series.

Other variants are the use of pulsed anaerobic digesters (Staldbauer *et al.*, 1991) or different combinations that can be included in parallel, staged or phased anaerobic processes.The relative importance of attachment versus suspended growth in the most important anaerobic reactors may be graded as follow (Iza, *et al.*, 1991):

$$FB > DSFF > AF > AF(hybrid) > UASB > Contact$$

The two first reactors, which have an industrial implantation, are specially representatives of the biofilm concept, because most of the biomass is retained by attaching.

2.2.1 *Fluidized Bed Reactors*. The FB technology offers many advantages to the process (Hickey, 1990) like: i) High concentration of biomass, attached to a heavy carrier, which can not be easily washed out from the reactor. ii) Very large surface area for biomass attachment. iii) Important dilution of the influent with the effluent, which provides alkalinity and, thus, some neutralization, reduces substrate concentration and contributes to reduce the shock effect of toxicant spikes. iv) High mass transfer properties. v) No channelling or gas hold-up. vi) Ability to control and optimize biofilm thickness and vii) Biomass carrier can be tailored to a specific application to enhance performance.

There are recent interesting papers on the design of these equipments treating global aspects (Fernandez-Polanco and Diez, 1988), or specific aspects (Hermanowicz and Cheng, 1990). The following steps in the design of these digesters are recommended (Iza, 1991):

a) Selection of support material and its physical (size, shape, density, hardness, specific area and roughness) and chemical characteristics (chemical inertia, adsorption properties).

b) Select the operating expansion and calculation of the liquid superficial velocity.

c) Bed (D, H) and equipment sizing, from the knowledge of fluidization characteristic, influent flowrate and concentration and an estimated hydraulic residence time. The H/D ratio is a very important parameter related with the recycling pumping that has economic implications, and it is normally established on the basis of a compromise between dilution factor and recycle factor.

d) Evaluation of the effects caused by biofilm growth, and

e) Impact of the gas production/release.

Most of studies using anaerobic expanded/fluidized reactors have reported the development of thin biofilms (Bull *et al.*, 1982, Switzenbaum and Eimstad, 1987). In a recent review (Hickey *et al.* 1991) it was analysed the most important factors affecting the biofilm development. Shear at both the macro and micro scales has a profound influence on film thickness. It is a consequence of the liquid flux rate, scale of the reactor, gas flux due to the gas production rate and organic loading rate. High shear will tend to keep the biomass thin, while higher organic loading rates will tend to foster development of thicker films.

Several industrial plants in operation are presented in table 4.

2.2.2 *Downflow Stationary Fixed Film Reactors (DSFFR).* Development of the DSFF reactor began in 1976 to solve the problems appeared during the treatment of wastewaters with high content of solids by using AF systems (Kennedy *et al.*, 1981).

The major design and operational differences between DSFF reactor and other fixed film reactors come from the use of an oriented packing material that forms vertical channels that run the length of the packing. Another difference is that in DSFF reactors wastewater enters always at the top of the reactor through a submerged distribution device and treated effluent is removed at the bottom. Recycling effluent to the influent can be used as required.

TABLE 4. FB Industrial plants

Company	Country	Volume (m^3)	Reference
Gist Brocades	Holland	4 x 300	Engler *et al.* 1986
"	France	2 x 125	Borhans *et al.* 1990
"	Germany	125	Borhans *et al.* 1990
Degremont	Spain	5 x 165	Oliva *et al.* 1990
"	France	--	Nicol *et al.* 1986
Reliance	India	850	Sutton *et al.* 1990
Envirex	USA	4 x 180	Sutton *et al.* 1990

In the DSFF reactor systems most of the active biomass is attached to the support media. A good adhesion of biomass to the support allows to work with high efficiency. Several factors have been studied to obtain the best adhesion conditions in order to increase the performance of these equipments: i) Type of support material: Although several controversial results appeared in literature it seems that needle punched polyester (NPP) and red drain tile clay have shown more rapid biofilm development than potter's clay, PVC or glass. All the factors above considered for the FB reactor would be considered in this section; ii) Kind of wastewaters; iii) Hydraulics of reactor.

The thickness of the mature biofilm developed varies not only with the kind of wastewater, but also with the loading rate applied. A biofilm thickness normally between 2-4 mm has been reported (Kennedy and van den Berg, 1982), although film thickness up to 5 mm have been observed (Hall, 1987)

The main goal of this kind of technology is its ability to treat high strength wastes with a high solids content. For more soluble wastes the attractiveness of the DSFF option declines. Other advantages of this technology are related with the nature of the stationary fixed biofilm developed, that appears to have a high stability. As a result, the DSFF reactor can withstand low temperatures, severe and repeated hydraulic overloadings, organic shock loads, sudden changes in waste composition, and starvation, with little or no effect on subsequent performance.

There are several large-scale industrial DSFF reactors working in different countries. The higher anaerobic digester in the world is a DSFF of 13.000 m^3 treating rum distillery wastes in Puerto Rico.

3. Acknowledgements

The authors would like to express their gratitude to the CICYT (Project BIO91-1153-C02-01)

4. References

Benefield, L.D. and Randall, C.W. (1987) 'Biological Process Design for wastewater treatment' InPrint, Inc. Virginia (USA).

Bovendeur, J. Spuij, F. Hermans, G.B. and Klapwijk, A. (1990) 'Mechanisms and kinetics of COD removal and oxygen consumption in a rotating biological contactor biofilm'. Wat. Sci Tech. 22(1/2) 53-73.

Bull, M.A. Sterritt, R.M. and Lester, J.N. (1982) 'The effect of organic loading on the performance of anaerobic fluidized beds treating high strength wastewater' Trans. Inst. Chem. Eng. 60, 373-376.

Capdeville, B. and Nguyen, M. (1990) 'Kinetics and modelling of aerobic and anaerobic film growth' Wat. Sci Tech. 22(1/2) 149-170.

Cooper, P.F. and Williams S.C. (1990) 'High rate nitrification in biological fluidized bed' Wat. Sci. Technol. 22(1/2), 431-42.

Crine, M. Schlitz, M and Vandevenne (1990) 'Evaluation of the performances of random plastic media in anaerobic trickling filters' Wat. Sci. Tech. 22(1/2) 227-238.

Eckenfelder, W.W. (1989) 'Industrial Water Pollution Control' 2nd Ed. Mcgraw-Hill Series in water resources and environmental engineering McGraw-Hill, New York (USA)

Enger, W.A. van Gils, W.M.A., Heijnen, J.J. and Koevoets, W.A.A. (1986) 'Full scale performance of a fluidized bed in a two-stage anaerobic wastewater treatment at Gist Brocades' In. Proc. NVA/EWPCA Water Treat. Conf. Anaerobic treatment: A grown-up technology, Amsterdam (The Netherlands). 299-306

Fenandez-Polanco, F. and Diez, V. (1988) 'Reactores anaerobios de lecho fluidizado. Diseño y operación' In: Depuración anaerobia de aguas residuales Secretariado de publicaciones. Universidad de Valladolid, Valladolid (Spain) 170-188.

Gujer, W. and Boller, M. (1990) 'A mathematical model for rotating biological contactors' Wat. Sci Tech. 22(1/2) 53-73.

Hall, E.R. (1987) 'Biofilm reactors in anaerobic wastewater treatment' Biotech. Adv. 5, 257-269

Harris, N.P. and and Hasford, G.S. (1976) 'A study of substrate removal in a microbial film reactor' Water Res. 10 935-943.

Henze, W. and Harremoens, P. (1983) 'Anaerobic treatment of wastewater in fixed film reactors - a literature review', Wat. Sci. Tech. 15(8/9), 1-101.

Hermanowicz, S.W. and Cheng, Y.W. (1990) 'Biological fluidized bed reactor: Hydrodynamic, biomass distribution and performance' Wat. Sci. Tech. 22(1/2), 193-202.

Hickey, R.F. (1990) 'Principles for use of adsorbant carriers in fluidized bed reactors'. In Seminar Biological fluidized beds for water and wastewater treatment: A user's forum. Ann Arbor, Michigan (USA) May 24-25.

Hickey, R.F. Wu, W.M., Veiga, M.C. and Jones, R. (1991) 'Star-up, operation, monitoring and control of high-rate anaerobic treatment systems' Wat. Sci. Tech. 24(8) 207-255.

Iza, J. (1991) 'Fluidized bed reactors for anaerobic wastewater treatment', Wat. Sci. Tech. 24(8), 109-132.

Iza, J. Colleran, E. Paris, J.M. and Wu, W.M. (1991) 'International workshop on anaerobic treatment technology for municipal and industrial wastewaters: summary paper' Wat. Sci. Tech. 24(8), 1-16.

Kennedy, K.J. van den Berg, L. and Murray, W.D. (1981) 'Advanced fixed film reactors for microbial production of methane from waste' In Proc 2nd World Congr. Chem. Eng. 1, 317-321.

Kennedy, K.J. and van den Berg, L. (1982) 'Continuous VS slug loading or downflow stationary fixed film reactors digesting piggery waste' Biotecnol. Letters 4, 137-142.

La Motta, E.J. (1976) 'Internal diffussion and reaction in biological films' Env. Sci. and Technol. 10(8), 765-769.

Lettinga, G. van Velsen, A.F.M. Hobma, S.W. de Zeeuw, W.J. and Klapwijk, A. (1980) 'Use of the Upflow Sludge Blanket (USB) reactor concept for biological wastewater treatment' Biotech. and Bioeng. 22, 699-734.

McDonald, D. V. (1990) 'Denitrification by fluidized biofilm reactor' Wat. Sci. Technol. 22(1/2), 451-461.

Martenson, L. and Frostell, B. (1982) 'Anaerobic wastewater treatment in a carrier assisted sludge bed reactor' in Proceedings of the IAWPRC-Specialized seminar on anaerobic treatment, June, Copenhagen (Denmark)

Nicol, R. and Prevot, C. (1986) 'Methanization of waste effluent from a dairy and brewery on a movile bed'. In Proc. NVA/EWPCA Water Treat. Conf. Anaerobic treatment: A grown-up technology, Amsterdam (The Netherlands)

Oliva, E., Jacquart, J.C. and Prevot, C. (1990) 'Fluidized bed reactors for anaerobic wastewater treatment', Wat. Sci. Tech. 22(1/2), 483-490.

Omura, T. Umita, T. Nenov, V. Aizawa, J. and Onuma M. (1991) 'Biological oxidation of ferrous iron in high acid mine drainage by fluidized bed reactor' Wat. Sci. Tech. 23(7/9) 1447-1456

Parker, D.S., Lutz, M.P. and Pratt, A.M. (1990) 'New trickling filter applications in the U.S.A.' Wat. Sci. Tech. 22(1/2) 215-226.

Stalbauer, E.A. Achenbach, R. Doll, D. Jehle, B. Jufner, B. Oey, L. and Quurck, J. (1991) 'Design and performance of pulsed anaerobic digesters' in Proc. 6th International symposium on anaerobic digestion. Sao Paulo (Brasil)

Sutton, P.M. and Mishra, P.N. (1990) 'Biological fluidized beds for water and wastewater treatment: A state of the art review' in: Proc. of the 1990 water pollution control federation conference, Washington, D. C. October 1990.

Switzenbaum, M.S. and Eimstad, R.B. (1987) 'Analysis of anaerobic biofilms' Env. Tech. Lett. 8, 21-32.

Veiga, M.C. (1989) 'Tratamiento por digestión anaerobia de los efluentes del procesado industrial de túnidos' Ph.D. dissertation. University of Santiago de Compostela (Spain)

Ware, A.J. Pescod, M.B. and Storch, B. (1990) 'Evaluation of alternatives to conventional disc support media for rotating biological contactors' Wat. Sci Tech. 22(1/2) 113-117.

Yang Zao-yan and Fan Zhen-san (1990) 'Treatment of leather and fur wastewater by a rotating biological contractor' Wat. Sci Tech. 22(1/2) 113-117.

DEVELOPMENT AND STABILITY OF BIOFILMS IN BIOREACTORS

M.C. VEIGA
Department of Industrial and Fundamental Chemistry
University of La Coruña
Campus da Zapateira, s/n
E-15071 La Coruña

R. MENDEZ and J.M. LEMA
Department of Chemical Engineering
University of Santiago de Compostela
Avenida de las Ciencias, s/n
E-15706-Santiago de Compostela

1. Introduction

Over the past few years the anaerobic digestion appeared as an alternative to aerobic digestion for the degradation of wastewaters with a high organic content. Consequently, a new generation of high-rate anaerobic reactors were developed. The performance of these advanced reactors depends on the retention of high bacterial concentrations. The prevailing mechanism is either the formation of bacterial aggregates with high settleable properties, the development of methanogenic biolayers at the surface of inert carriers or both (Hickey *et al.*, 1991). In the present case, we will focus on the anaerobic reactors where the biomass is retained by adhesion to a support medium, although in some of them the biomass can also been trapped in the interstitial pore volume of the support media: anaerobic filter (AF), downflow stationary fixed film (DSFF) and fluidized bed reactor (FBR).

Unfortunately, anaerobic fixed film reactors may require a long start-up time before reaching efficient and stable operation conditions. The long start-up times are mainly dependent on the slow accumulation of anaerobic biofilms on the media in the reactor. The process of biofilm formation is not well understood (Characklis, 1982; Savage and Fletcher, 1985). Trulear and Characklis (1982) postulated that several sequential processes are involved in the development of a mature biofilm on support media:

1. Transport and adsorption of organic molecules to the surface.
2. Transport of microbial cells to the surface.
3. Microorganism attachment to the surface.
4. Microbial transformations (growth and exopolymer production) at the surface resulting in the production of biofilm, and
5. Partial detachment of biofilm, at a rate approximately equal to growth and additional attachment (steady state).

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 421–434.

The first three processes listed above occur relatively quickly (hours). Unfortunately the fourth process occurs rather slowly, mainly due to the slow growth rates of anaerobic bacteria, specifically the methanogenic bacteria.

There are several factors affecting bacterial attachment to a surface (Daniels, 1980; Salkinoja-Salonen *et al.*, 1983), including the characteristics of the microorganisms, support surface and surrounding environment. It should be taken into account that all of the environmental and operating factors act together and can influence each other. There appears to be interactions between support surfaces and reactor.

The main factors influencing the initial biofilm development in bioreactors are discussed below. The effect of some of these factors on the stability of biofilms in fixed or fluidized bed bioreactors will also be considered. Moreover, as the biofilm accumulates changes in its properties may occur.

2. Factors influencing biofilm development

2.1. WASTEWATER CHARACTERISTICS

2.1.1. *Wastewater composition.* The nature of the substrate strongly affects the production of bacterial exopolysaccharides which are considered to play an important role in the biofilm formation. Carbohydrate wastewaters stimulate the growth of biofilms on the support media because they promote the polysaccharide production (Wilkinson, 1958; Veiga *et al.*, 1990).

Table 1. Anaerobic Fixed Film Reactor Studies.

Reactor type	Wastewater g COD/L	Temp. °C	Support	Volumetric load rate g COD/m^3.d	Methanogenic activity[a] g COD/g VSS.d	Biomass on support g VSS/m^2	Biomass on carrier g VSS/kg	Ref.
DSFF	Lactose	37	clay ring	2	0.6	25/25/25		(1)
DSFF	Tuna	37	clay ring	3	0.35	9.8/-/7.3		(2)
AF	Mussel	37	PVC ring	24	0.6 - 0.9	36/29/29		(3)
AF	Mussel	55	PVC ring	12	0.8	38/53/65		(3)
FB	VFA	37	sand	37 - 147	5.1		220/46/-	(4)
FB	Whey	35	sand	13.4 - 37.6	-		-	(5)
FB	Sewage	15 - 25	activated carbon	1.8 - 3.6	-		-	(5)
FB	Yeast	37	sand	20 - 60	-		-	(5)
AF	Slaughterhouse	37	PVC ring	6	0.45	10/13/15	-	(6)

[a] Average values for samples from top, middle and bottom sludge layer.

References:

(1) Pan, M. (1991); (2) Veiga, M.C. (1989); (3) Soto *et al.*, (1992a); (4) Gorris *et al.*, (1989); (5) Bonastre and Paris, (1989); Santiago, P. (1992).

A list of several anaerobic studies treating different wastes, is provided in Table 1.

In some cases the biofilm formation has been accelerated by starting-up the reactor with synthetic feeds, gradually replacing them with the waste to be treated (Dennis and Jennett, 1974; Lovan and Foree, 1971). The growth of polysaccharide-producing methanogenic bacteria may be stimulated by the addition of a substrate that may be directly metabolized, such as methanol (Tait and Friedman, 1980; Bull *et al.*, 1983; Stephenson andLester, 1986). The addition of methanol to the wastewater increases the C:N ratio which encourages the production of extracellular polysaccharides and favors bacterial attachment to the support material (Wilkinson, 1958). A reduction in the start-up time was also observed when a wastewater containing a low C:N ratio was supplemented with lactose (Veiga *et al.*, 1990).

2.1.2. *Nutrients*. The waste should contain, in an available form, all the essential nutrients required for anaerobic growth. It may be necessary to supplement the waste stream with nutrients which appear to be deficient (Lauwers *et al.*, 1989).

A COD:N:P ratio in the range 100:(10-1):(5-1) is recommended (Weiland and Rozzi, 1991).

Mendez *et al.* (1989) studied the influence of a low level of essential nutrients (nitrogen and phosphorus) on the biofilm formation and on the methanogenic activity of the biomass present on that biofilm. The experiments were carried out with a DSFF reactor using clay as a support material and fed with an artificially prepared waste water containing lactose as carbon source. Substrate mixtures with different C:N:P ratios were fed to each reactor. The assumed balanced substrate mixture was characterized by a C:N:P ratio of 250:7.5:1. For the nitrogen and the phosphorous deficient media these ratios were respectively 250:1.5:1 and 250:7.5:0.2. In all cases, the reactors presented similar patterns respect to biogas yield and COD efficiency for an organic loading rate of 2 $Kg/m^3.d$. However, when pulses of individual volatile fatty acids and of lactose were applied, the kinetics of degradation were different, being the acetate and propionate degradation rates lower in the reactor fed with a nitrogen deficient substrate, which could mean that a more equilibrated population developed in the digester with the balanced substrate, being the acetate and propionate consuming bacteria more affected by the low level of nitrogen. All three reactors presented the same concentration of biomass in suspension but the concentration of attached biomass varied with the substrate used to feed the reactor and with the height in each reactor. The nitrogen deficient reactor presented the highest concentration of biomass attached to the support, followed by the balanced reactor (Fig. 1). This fact, may be an indication that a low concentration of nitrogen in the feed could enhance the biopolymer production by the methanogenic bacteria. This was also reported by Veiga *et al.* (1991). They observed that the exopolymer production by *Methanobacterium formicicum* was stimulated by a limitation in some nutrients, showing that the carbon utilization shifts towards exopolysaccharides production when the C:N and/or C:P ratios are high.

The biomass concentration was higher in the lower part of the reactor (Fig. 1), although the percentage of volatile matter was higher in the upper part, except in the phosphorus deficient reactor where it remained constant (Fig. 2). This could be related to the fact that the fresh feed enters at the top of the reactor.

The biomass of the biofilm from the nitrogen deficient and the phosphorus deficient reactors presented a relative specific activity of 32 and 82%, respectively, compared to the balanced reactor. The bacterial concentration in the liquid in the balanced substrate digester was lower than in the other two reactors. Futhermore, as described below, the microbial populations varied with the type of substrate used. The balanced reactor contained mainly isolated cocci, whereas the digester with a low level of nitrogen contained basically agroupated cocci, and the biomass from the third digestor contained mainly filamentous bacteria.The wastewater should contain a properly balanced trace elements concentration of cations such as iron, nickel, cobalt

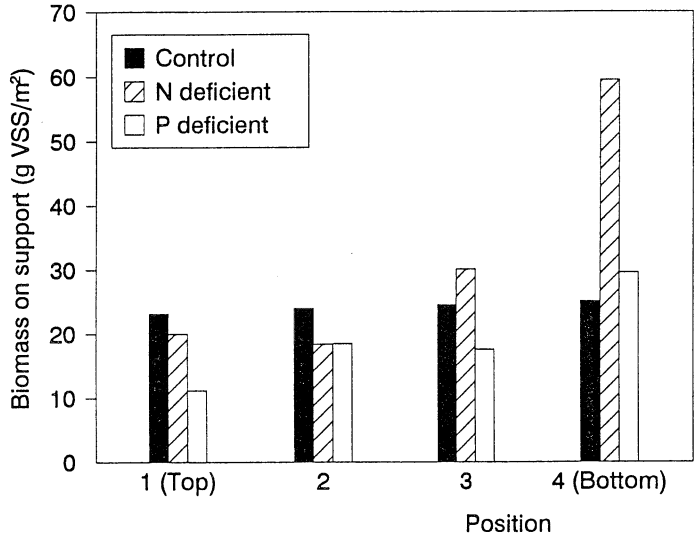


Figure 1. Biomass distribution on clay in three DSFF reactors with different C:N:P ratios, fed with synthetic wastewater containing lactose (Pan, 1991).

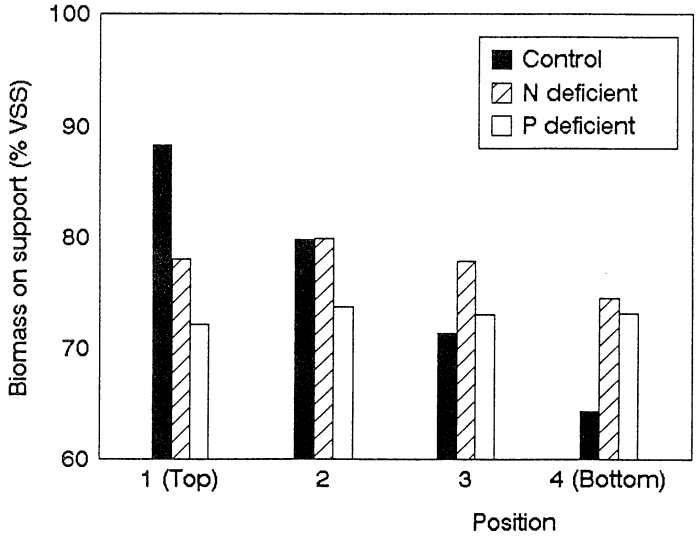


Figure 2. Organic content (% VSS) of biofilms from three DSFF reactors with different C:N:P ratios, fed with synthetic wastewater containing lactose (Pan 1991).

and molybdenum. For example, Kelly and Switzenbaum (1984) were not able to establish a mature biofilm in an anaerobic fluidized bed reactor treating whey until Ni, Co and Fe salts were added to the feed. Murray and van den Berg (1981) showed that trace of nutrient addition allowed accumulation of a thicker methanogenic fixed film.

2.1.3. *Environmental conditions*. The pH of the medium should be maintained as close as possible to neutrality. If necessary, a pH decrease due to the activity of acidogens may be prevented by the addition of alkalinity.

Diaz-Baez (1988) observed that the growth of methanogenic bacteria is influenced by the anaerobic conditions and the redox potential which should be maintained low enough in order to increase the growth rate of methanogenic bacteria.

Soto *et al.* (1992a) determined the distribution of biomass and the methanogenic and non-methanogenic activities of the sludge present in two identical mesophilic and thermophilic anaerobic filters. The methanogenic activities were similar for both reactors, ranging from 0.6 to 0.9 kgCOD/kg VSS.d. In the thermophilic reactor most of the biomass was attached to the support medium whereas in the mesophilic one the biomass was mainly occluded (Fig. 3). The distribution of attached biomass was more homogeneous in the mesophilic than in the thermophilic reactor. The biofilm thickness in both reactors varied along the reactor, from 0.8 to 2.8 mm, getting thicker towards the upper part of the reactor.

Absence of inhibitory concentrations of toxic compounds is required for a good biofilm development.

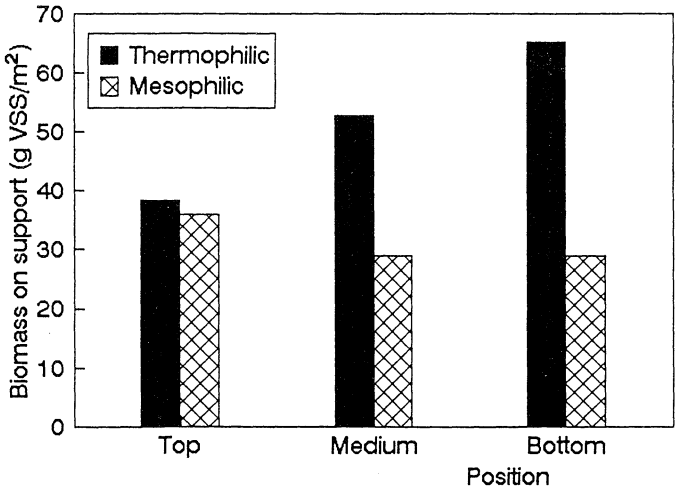


Fig. 3. Biomass distribution on the support media in the mesophilic and thermophilic reactors (Soto *et al.*, 1992a).

2.2. OPERATIONAL FACTORS

2.2.1. *Inoculation.* It is recommended to inoculate the reactor with a rich inoculum from a working reactor treating a similar waste, when available. Such an inoculum offers the advantage of being already acclimated to components of the waste being treated and also to any toxic or inhibitory compounds which might be present. Salkinoja-Salonen (1983) suggested a seed inoculum of 30-50% of the reactor volume to speed up the start-up period.

Shapiro and Switzenbaum (1984) showed that the initial rate of methanogens accumulation and anaerobic biofilm accumulation might be increased by maintaining a large concentration of microorganisms in the bulk liquid.

According to Gorris *et al.* (1989) the type of inoculum did not significantly affect the course of biofilm formation or the biofilm composition during the start-up of fluidized bed reactors treating VFA waste. However, according to Dunn and Petrozzi (1991) the inoculum seems to have the greatest influence on loading, residence time and carrier type during the start-up of a FB reactor.

The propensity of microorganisms for attachment has also to be considered. In the case of aerobic bacteria, some organisms have a greater tendency for attachment than others. Isa *et al.* (1986) observed that methane producing bacteria are able to colonize and adhere to polyurethane carriers better than sulfate reducing bacteria. Thus the methane producing bacteria were able to outcompete the faster growing sulfate reducing bacteria by their higher propensity for attachment.

The growth phase of the organisms as well as their actual number may affect the biofilm development. Some researchers observed during studies under aerobic conditions that the rate of attachment of microorganisms to the support depending on the physiological state decreased in the following order: exponential phase > stationary phase > death phase (Fletcher, 1977; Marshall *et al.* 1971).

2.2.2. *Organic loading rate.* It has been recommended to keep a low organic loading rate (less than 0.1 kg COD/kg VSS.d) and a low feed to seed ratio (biomass density kept over 20 kg VSS/m^3 during the start-up time (Henze and Harremoes, 1983; Salkinoja-Salonen *et al.*, 1983).

Several authors reported that the initial use of a low organic loading rate gradually increased until reaching the operating reactor loading was beneficial for a faster start-up (Bull *et al.* 1983; de Zeeuw *et al.*,1982).

It has also been shown beneficial to maintain a low volatile acids production and to prevent pH depression.

Kennedy and Droste (1985) reported that the methanogenic activity decreased with higher organic loading rates during the start-up period. It is not clear whether this is a temporary or a permanent phenomenon. Henze and Harremoes (1983) concluded that the organic loading rate should be increased by 50% per week.

Contrary to other authors, Shapiro and Switzenbaum (1984) showed that anaerobic biofilm accumulation increased with increasing organic space loading in a well buffered system. Higher organic space loading provides a large volatile suspended solids concentration in the bulk during the start-up period and, in general, it has been shown that an increase in microbial concentration results in an increase in the number of bacteria attaching to a surface (Fletcher, 1977; Bryers and Characklis, 1981).

2.2.3. *Hydrodynamics.* Good mixing characteristics are important to form an active and uniform biofilm. These characteristics are influenced by media size, type and placement in AF. Recycle is often employed to improve mixing (Samson *et al.*, 1985). In the FBR, recycle is generally required to obtain sufficient upflow velocities. Furthermore, a larger biofilm formation was observed at higher velocities. This effect appears because more substrate can be

transported from the bulk liquid to the biofilm at higher fluid velocities. Characklis (1981) reported that the biofilm adheres more firmly when it grows under high shear stress.

2.2.4. *Addition of polymers*. The addition of chemical polymers might be used to modify the surface characteristics of the support media. Their presence could increase the probability of microbial attachment by amplifying the area and the amount of adsorption sites.

Precoating the support surfaces with synthetic cationic polymers enhances the initial biofilm accumulation with aerobic mixed cultures and nitrifying bacterial cultures. Switzenbaum *et al.*, (1987b) studied the influence of different polymers for precoating surfaces but none of them appeared to enhance biofilm development.

2.3. SUPPORT MEDIA

2.3.1. *Anaerobic Filters Systems*. The propensity of microorganisms to attach to solid surfaces may vary significantly depending on the type of material used. Therefore the material to which methanogenic and anaerobic bacteria have a high affinity for adsorption are favorable for biofilm development. Sometimes the results reported about biofilm formation in the support material are contradictory. This fact may be explained by different operating conditions, wastewater composition or type of reactor.

In anaerobic filters, the chemical oxygen demand removal efficiency at steady state conditions appears to be governed by the upflow velocity through the void space within the material (Young and Danhab, 1982) and bacteria are mainly retained in suspended form. Therefore bacteria retention seems to be as related to media shape and void size as to unit surface area (Young and Danhab, 1983).

Wilkie and Colleran (1984) observed in an anaerobic filter treating pig slurry that the type of support media used can dramatically reduce the start-up time. The rate of biomass accumulation was higher in a filter filled with clay fragments than in a filter packed with plastic pallrings, coral or mussel shells but the performance of all reactors at steady-state were similar. One possible explanation for the results obtained with clay is the leaching of minerals, nutrients or both (Huysman *et al.*, 1983).

Switzenbaum *et al.* (1987b) observed a greater biofilm formation in stainless steel and teflon media than in PVC and aluminum.

Contrary to the AF, DSFF reactors the support material affects both the rate of biofilm development and the maximum performance at steady-state.

Veiga *et al.* (1992a) compared the adhesion of biomass for three different support media (clay, polystyrene and polyvinyl chloride) from a DSFF reactor treating a tuna processing wastewater, which posesses a relatively high protein content. An uniform and slime film was distributed along the three support materials. Higher biofilm development was observed for polystyrene and then for clay.

Kennedy and Droste (1985) observed among five different support media that needle punched polyester (NPP) and red drain tile clay allowed higher biofilm formation. Materials with roughened surfaces allowed the best start-up. The performance appeared to be a function of the ease with which bacteria become entrapped and attached. Similarly to Wilkie *et al.* (1984), they also found that in the case of clay, leaching of minerals out of the clay may be a favorable factor. They observed that the start-up with high surface area to volume ratio material required a longer period of time and that rates of biofilm development was faster for lower strength wastes. The porous and rough surface allowed organisms to be entrapped and protected those which adhere to the surface.

Verrier *et al.* (1988) reported that the hydrophobicity of the support material affects the adhesion of methanogenic bacteria and initial biofilm development of anaerobic biofilms.

2.3.2. *Anaerobic Fluidized Bed Reactors*. In fluidized bed reactors, the biomass is retained as a biolayer covering small carriers such as sand, AlO_3, active charcoal and synthetic resins, Sand is the most often used in FBR. High biomass concentration can be retained with this material (Heijnen *et al.*, 1986).

Important factors that may influence the biofilm development and to be considered for the design of the reactor are size, shape, density, hardness, specific area and roughness of the support media (Iza, 1991).

Salkinoja-Salonen *et al.* (1983) showed that when the FBR works at long hidraulic retention time (HRT) the biomass is present both in suspension and as a biofilm. The suspended bacteria are mainly acidogens (Bull *et al.*, 1984) and most of the methanogens are attached to the surface of the carrier. At short HRT, the biomass grows mainly in attached form, the suspended bacteria are washed out and the start-up of the reactor is faster (Heijnen *et al.* 1986).

One of the advantages of FBR is the large surface available for adhesion, allowing a high concentration of biomass. The use of porous materials present also the advantage of internal pores which can be colonized.

Poels *et al.* (1984) found the highly porous poly-urethane foam sponges able to be colonized by methanogenic cultures in a completely mixed reactor treating piggery manure. Huysman *et al.* (1983) compared the colonization of porous and non porous packing materials. He observed that the most important factors for biofilm development were surface roughness for non porous materials while for porous materials the size of the pore sand the degree of porosity had the greatest influence.

In general, rough surface nonporous materials and porous materials were found to be favorable for the biofilm development. In the case of porous materials, the size of the pores and the degree of porosity are the most important characteristics.

Properties such as specific surface and surface charge relate to phenomena at the molecular and ionic level. They do not represent the cation exchange capacity or the surface available to the average sized microorganisms. Therefore their possible role in the adhesion of microorganisms must be considered with care (Huysman *et al.*, 1983).

3. Biofilm characteristics

There are numerous reports proving the formation of biolayers on carrier materials during operation of anaerobic filters or fluidized bed reactors treating different types of wastewaters (Robinson *et al.* 1984; Denac and Dunn, 1987; Pan, 1991; Soto *et al.*, 1992a, Veiga *et al.*, 1992a). However, very little detailed information is available concerning the nature of biofilms.

The biofilms are formed by a wide diversity of organisms. In general, the biofilms are thin and dense, with rough uneven surfaces and contain channels and holes. A large amount of cell and inorganic debris are observed in the films. The morphology may differ among different systems or among different kinds of feed (Switzenbaum & Eimstad, 1987a).

3.1. PHYSICAL AND CHEMICAL CHARACTERISTICS

Biofilms found on the various supports do not differ significantly in microbial content or overall aspect. The thickness of the mature biofilm varies with the loading rate and the substrate fed to the reactor. The biofilms from AF and DSFF reactors are 1 to 3 mm thick and they display a rough and uneven surface (Robinson *et al.*, 1984). Hall (1987) reported a biofilm thickness of up to 5 mm.

Scanning electron microscopy of the biofilms revealed their rough surface with numerous and variable sized vents. It is believed that these vents allow biogas release and possibly serve as

conducts for importing nutrients to the interior of the granules. Robinson *et al.* (1984) reported that the vents had a diameter of 100-500 μm.

Inert suspended solids may be transported to the biofilm and incorporated into its structure. The mineral composition of the biofilm varies with the chemical composition of the bulk liquid, while the carbon and energy source influence the organic composition of the biofilm. About 39-58% of biofilm may be inorganic material. The accumulation usually increases with increasing loading rates. Biofilms from reactors treating industrial wastewaters generally have higher ash contents than those from synthetic wastes. Generally, the biofilms from the lower part of the reactors contain higher ash levels than that from the upper part of the bed (Veiga,1989; Pan, 1991; Soto *et al.*, 1992a).

Many mineral precipitates containing Ca, Mg and P are embedded in the biofilm (Robinson *et al.*, 1984). A higher concentration of material is present towards the base of the film, lower layers being characterized by the presence of a thick matrix. The latter is loosely bound to an underlying mineral deposit which adheres tightly to the surface of plastic materials and contains mainly Ca and P.

In a FBR, the biofilm thickness is one of the most important parameters affecting the overall performance of the reactor (Shieh and Keenan, 1986). In an annular reactor, the thickness depends on the substrate loading rate (Characklis, 1981). The mass transfer kinetics of the substrate from the bulk medium into the biofilm is dependant on its thickness and it was observed that bigger biofilms do not necessarily present a faster degradation than thinner biofilms. This thickness can be maintained in a FBR by controlling the fluidized bed height and by regularly washing the excess biomass (Shieh and Keenan, 1986). Furthermore, an excessive accumulation of biofilm on the fluidized medium leads to a greater bed height increase which reduces the bed stability against hydraulic load variations. In some cases, bioparticles can be washed-out from the reactor. In anaerobic reactors the biofilm thickness varies from 6 to 200 μm for carrier particles of 0.3 to 0.6 mm (Switzenbaum and Jewell, 1980). Among other factors, the carrier diameter affects the biofilm thickness. In the anaerobic fluidized bed reactors, a difference with the aerobic reactors, it is not usually required to control the biofilm thickness, because the gas flow or the liquid velocities is enough to compensate the biomass growth. In the case of wastewaters with a high carbohydrate content, such as whey, it may be necessary to remove some biomass from the reactor (Boering and Larsen, 1982).

3.2. MICROBIAL COMPOSITION AND STRUCTURE

The initial organism attachment may occur in a relatively short time. The bacterial population is quite heterogeneous but methanogens, mainly *Methanosarcina spp.* and *Methanosaeta spp.*, are the prevailing microorganisms (Robinson *et al.*, 1984). The slow accumulation of anaerobic biofilms which has been reported is most likely due to the slow growth rates of methanogens.

The type of microbial populations present in a biofilm depends on the reactor configuration. Different mechanistic influences selected for different methanogenic associations (Switzembaum *et al.*, 1987). Methanogenic rods were found dominating in a sedimentation type system (granules), while methanogenic cocci in an adhesion type system (fixed film). More sarcina was observed in the high shear anaerobic fluidized bed than in the low shear anaerobic filter and the anaerobic upflow sludge blanket reactor. In the latter two reactors, rod type organisms were more numerous than sarcina.

Mendez *et al.* (1989) observed that the population of microorganisms present in the biofilm of a DSFF reactor treating lactose varied with the C:N:P ratios. The nature of the substrate influences also the microbial population present in the biofilm. Veiga *et al.* (1992a) observed that *Methanogenium spp., Methanosarcina spp., and Methanobacterium spp.*, were the prevailing methanogens present in the biofilm of a DSFFR fed with a tuna processing wastewater. This wastewater was characterized by high protein and salt contents. The presence

of *Methanogenium spp.* is unusual in anaerobic digesters and is most probably related to the relatively high salinity of the medium.

Van Loosdrecht *et al.* (1987) observed that cell surface characteristics determining adhesion are influenced by growth conditions. They found that bacteria become more hydrophobic during the exponential phase of growth.

3.3. EXTRACELLULAR POLYMERS

The production of extracellular polymers (ECP) is a relevant factor in the initial bacterial adhesion to surfaces (Costerton *et al.*, 1985) and in the formation of a stable biofilm (Christensen and Characklis, 1989). The ECP have been observed in different types of biofilms by scanning electron microscopy and by transmission electron microscopy (Robinson *et al.*,1984). It has been demonstrated that bacterial exopolymers contribute to the adhesion between different species of methanogens and other syntrophic acetogenic species present in granules, improving their long-termstability (Alibhai and Foster, 1986) and facilitating the process of interspecies electron transfer. Ross (1984) observed that ECP accumulation allows the formation of clumps by bacteria. Several factors such as changes in temperature, substrate, nutritional balance and/or the diversity of microflora may influence the production of ECP (Alibhai and Foster, 1986; Veiga *et al.*, 1991).

Some authors have studied the exopolymers production and their composition. Dolfing *et al.* (1985) determined their composition in different anaerobic sludges. Brown and Lester (1980) analyzed the composition of exopolymers present in aerobic activated sludges.

Robinson *et al.* (1984) noted that the methanogens play an important role in the formation of biofilms. Veiga *et al.* (1991, 1992b) determined the amount and composition of exopolymers produced by methanogens in pure culture. They found that the exopolymer was formed mainly of polysaccharides.

4. Stability of biofilms

The factors influencing the stability of the digesters, may also affect the stability of the biofilm. In general, those factors can be divided according to their short- or long-term effect (Soto et al., 1992b). Some of the factors that produce a suddenly desestabilization of the reactor (effect on a short-term basis) are: shocks of organic loading rate, of hydraulic loading rate and of temperature and an unexpected presence of toxic compounds in the feed. Usually, the reactors recover quite well after a desestabilization produced by the above mentioned factors, and the biofilm is practically not affected by those circumstances. However, the long-term factors are more related to the activity and stability of biofilms. These long-term factors are: loss of biomass from the reactor, decrease of the biomass specific activity and problems of mass transfer.

4.1. LOSS OF BIOFILM

High superficial velocities may produce a sloughing of sections of biofilm from the reactor. In the case of FBR high liquid velocities are recomended in order to eliminate the biomass in suspension and to favour the formation of biofilms. However, the velocity has to be controlled, according to the type of substrate fed to the reactor, to obtain the appropiate biofilm thickness and to avoid shear detachment.

The presence of toxic compounds in the influent may also provoke a detachment of the biofilm from the carrier or support material.

4.2. LOSS OF SPECIFIC ACTIVITY

The activity of the biomass can decrease during the operation of the reactor. This loss of activity can be related to: an accumulation of inorganic material, an accumulation of organic material no biodegradable and due also to the presence of toxic compounds. Usually, a high concentration of inorganic material on the biofilm is observed when the wastewater treated contains an important fraction of inorganic material. The accumulation of organic material on the biofilm is also related to the wastewater fed to the reactor. For instance, proteinaceous wastewaters, such as slaughterhouse wastewater, normally present an organic fraction difficult to degrade in an anaerobic digester (Santiago, 1992). This loss of activity can be prevented by a previous separation of those organic and inorganic fractions present in the feed. A decrease of the activity can also be related to the presence of toxic compounds. In some cases, and depending on the concentration and nature of toxic compounds, an adaptation of the microorganisms to the toxic compounds can take place. For example, Feijoo (1991) observed an adaptation of the microorganisms to high concentrations of sodium, up to 9 g Na^+/L. He also observed that the toxicity effect of Na^+ is different depending on the substrate fed to the digester, and that it can be reduced due to the antagonistic effect of different cations present in the wastewater.

Another factor influencing the stability of the biofilm, is associated with problems of mass transfer. An excesive accumulation of material may also cause a diminution of the diffusion of molecules through the biofilm, creating a gradient across the biofilm and decreasing its activity and stability. The accumulation of biofilm vary with the type of wastewater, wastewaters with a high carbohydrate content allow a thicker biofilm development. The thickness of biofilm might be controlled by wasting biomass from the reactor or by increasing the superficial velocities (by decreasing the HRT or by increasing the recirculation rate).

5. Acknowledgements

This work was partially founded by a grant from the DGICYT (Project No. PB89-0555).

6. References

Alibhai, K.R.R. and Foster, C.F. (1986) 'An examination of the granulation process in UASB reactors', Environ. Technol. Lett., 7, 193-200.

Bull, M.A., Sterritt, R.M. and Lester, J.N. (1983) 'An evaluation of four start-up regimes for anaerobic fluidized bed reactors', Biotechnol. Lett.,5, 333-338.

Bryers, J.P. and Characklis, W.H. (1981) 'Early fouling formation in a turbulent flow system: over all kinetics', Water Res., 15, 483-491.

Boering, P.H. and Larsen, V.F. (1982) 'Anaerobic fluidized bed whey treatment', Biotechnol. Bioeng., 24, 2539.

Bonastre, N. and Paris, J.M. (1989) 'Survey of laboratory, pilot and industrial anaerobic filter installations', Process Biochem., February, 15-20.

Brown, M.J. and Lester, J.N. (1980) 'Comparison of bacterial extracellular polymer extraction methods', Appl. Environ. Microbiol., 40, 179-185.

Bull, M.A., Sterrit, A.M. and Lester, J.N. (1984) 'The distribution of bacterial activity in an anaerobic fluidized bed reactor', Water Res., 18, 1017-1020.

Characklis, W.G. (1981) 'Fouling biofilm development: a process analysis', Biotechnol. Bioeng., 23, 1923-1960.

Christensen, B.E., Characklis, W.G. (1989) 'Physical and chemical properties of biofilms', In: Characklis W.G. and Marshall, K.C. (eds.), Biofilms, Wiley, New York, pp. 93-130.

Costerton, J.W., Marrie, T.J., Cheng, K.J. (1985) 'Phenomena of bacterial adhesion'. In: Savage, D.C. and Fletcher, M. (eds.), Bacterial adhesion, mechanisms and physio-logical significance, Plenum Press, New York, pp. 3-43.

Daniels, S.L. (1980) 'Mechanisms involved in sorption of microorganisms to solid surfaces'. In: Bitton, G. and Marshall, K.C. (eds.), Adsorption of microorganisms to surfaces, John Wiley and Sons, N.Y., pp. 7-58.

Denac, M. and Dunn, I.J. (1988) 'Packed and fluidized-bed biofilm reactor performance for anaerobic wastewater treatment', Biotechnol. Bioeng., 32, 159-173.

Dennis, N.D. and Jennett, J.C. (1974) 'Pharmaceutical waste treatment with an anaerobic filter', Proc. Purdue Industrial Waste Conference, Ann Arbor Science, Ann Arbor, MI, pp. 29-36.

Diaz-Baez, M.C. (1988) 'A study of factors affecting attachment in the start-up and operation of anaerobic fluidized bed reactors'. In: A. Tilcheand A. Rozzi (eds.), Fifth International Symp.Anaerobic Digestion, Monduzzi Editore, Bologna, Italy, pp. 105-108.

Dolfing, J., Griffioen, A., van Neerven, A.R.W. and Zevenhuizen, L.P.T.M.(1985) 'Chemical and bacteriological composition of granular methanogenic sludge', Can. J. Microbiol., 31, 744 -750.

Dunn, I.J. and Petrozzi, S. (1991) 'Start-up of anaerobic biofilm fluidized bed reactors on molasses and phenol under various conditions', Bioprocess Engineering, 6, 21-28.

Feijoo, G. (1991) 'Toxicidade por sodio en procesos de dixestion anaerobia: fenómenos de antagonismo e adaptación'. M.Sc. dissertation, Santiago de Compostela, Spain.

Fletcher, M. (1977) 'The effects of culture concentration, age, time and temperature on bacterial attachment to polystyrene', Can. J. Microbiol., 23, 1-6. Gorris, L.G.M., van Deursen, J.M.A., van der Drift, C. and Vogels, G.D. (1989) 'Biofilm development in laboratory methanogenic fluidized bed reactors', Biotechnol. Bioeng., 33, 687-693.

Hall, E.R. (1987) 'Biofilm reactors in anaerobic wastewater treatment', Biotechnol. Adv., 5, 257-269.

Henze, M. and Harremoes, P.L. (1983) 'Anaerobic treatment of wastewater in fixed-film reactors', Water Sci. Technol., 15, 1-101.

Heijnen, J.J., Mulder, A., Enger, W. and Hoeks, F. (1986) 'Review of the application of anaerobic fluidized bed reactors in waste water treatment', In: Anaerobic treatment a grown up technology', Industrial presentation group, Schiedam, The Netherlands, pp. 159-174.

Hickey, R.F., Wu, W.M., Veiga, M.C. and Jones, R. (1991) 'Start-up, operation, monitoring and control of high-rate anaerobic treatment systems', Water Sci. Technol., 24, 207-255.

Huysman, P., Meenen, P., van Assche, P., and Verstraete, W. (1983) 'Factors affecting the colonization of non porous and porous packing materials in model upflow methane reactors', Biotechnol. Lett., 5, 643-648.

Isa, Z., Grusenmeyer, S. and Verstraete, W. (1986) 'Sulfate reduction relative to methane production in high rate anaerobic digestion: microbiological aspects', Appl. Environ. Microbiol., 51, 580-587.

Iza, J. (1991) 'Fluidized bed reactors for anaerobic wastewater treatment', Wat. Sci. Technol., 24, 109-132.

Kelly, C.R. and Switzenbaum, M.S. (1984) 'Anaerobic treatment: temperature and nutrient effects', Agric. Wastes, 10, 135.

Kennedy, M. and Droste, R.L. (1985) 'Start-up of anaerobic downflow stationary fixed film (DSFF) reactor', Biotechnol. Bioeng., 27, 1152-1165.

Lauwers, A.M., Heinen, W., Gorris, L.G.M. and Van der Drift, C. (1989) 'Variation of parameters affecting the start-up of methanogenic fluidized bed reactors', Biotechnol. Lett., 11(12), 907-912.

Lovan, C.R. and Foree, E.G. (1971) 'The anaerobic filter for the treatment of brewery press liquor waste', Proc. Purdue Industrial Waste Conference, 20, 1074.
Marshall, K.C., Stout, R. and Mitchell, R. (1971) 'Mechanism of the initial events in the sorption of marine bacteria to surfaces', J. Gen. Microbiol., 68, 337-348.
Mendez, R., Pan, M. and Lema, J.M. (1989) 'Effect of C/N/P ratio on the performance of a DSFFR working at low organic loading rates', Wat. Sci.Technol., 21, 1673-1676.
Murray, W.D. and and van den Berg, L. (19810) 'Effects of nickel, cobalt and molybdenum on performance of methanogenic fixed-film reactors', Appl. Environ. Microbiol., 42, 502-505.
Pan, M. (1991) 'Tratamiento de efluentes de industrias lácteas en reactores anaerobios de alta carga: Influencia de la relación C/N/P y del equipo', Ph.D. Dissertation, University of Santiago de Compostela, Spain.
Poels, J., van Assche, P. and Verstraete. W. (1984) 'High rate anaerobic digestion of piggery manure with polyurethane sponges as a support material', Biotechnol. Lett., 6, 747-752.
Robinson, R.W., Akin, D.E., Nordstedt, R.A., Thomas, M.V. and Aldrich,H.C. (1984) 'Light and electron microscopic examinations of methano-producing biofilms from anaerobic fixed-bed reactors', Appl. Environ. Microbiol., 48, 127-136.
Ross, W.R. (1984) 'The phenomenon of sludge pelletization in the treatmentof maize processing waste', Waste SA, 10,197-204.
Salkinoja-Salonen, M.S., Nyns, E.J., Sutton, P.M., van den Berg, L. and Wheathey, A.D. (1983) 'Start-up of an anaerobic fixed-film reactor', Wat.Sci.Technol., 15, 305-308.
Samson, R., van den Berg, L. and Kennedy, K.J. (1985) 'Mixing characteristics and start-up of anaerobic stationary fixed film (DSFF) reactors', Biotechnol. Bioeng., 27, 10-19.
Santiago, P. (1992) 'Tratamento de augas residuais de matadoiros mediante filtro anaerobio', M.Sc. dissertation, University of A Coruña, Spain.
Savage, D.W. and Fletcher, M. (Eds.) (1985) Bacterial adhesion, Plenum Press, London.
Shapiro, M. and Switzenbaum, S. (1984) 'Initial anaerobic biofilm development', Biotechnol. Lett., 6, 729-734.
Shieh, W.K. and Keenan, J.D. (1986) 'Fluidized bed biofilm reactor for wastewater treatment', In: A. Fletcher (ed.), Advances in biochemical engineering/Biotechnology, Springer-Verlag Berlin Heidelberg, 33,pp. 131¬169.
Soto, M., Mendez, R. and Lema, J.M. (1992a) 'Characterization and comparison of biomass from mesophilic and thermophilic fixed bed anaerobic disgesters', Wat. Sci. Technol., (in press).
Soto, M., Mendez, R. and Lema, J.M. (1992b) 'Diseño y operación de digestores anaerobios. II.Operacion y control'. Ingeniería Química (in press).
Stephenson, T. and Lester, J.N. (1986) 'Evaluation of start-up and operation of four anaerobic processes treating a synthetic meat waste', Biotechnol. Bioeng., 28, 372-380.
Switzenbaum, M.S. and Jewell, W.I. (1980) 'Anaerobic attached film expanded bed reactor treatment', JWPCF, 52, 1953.
Switzenbaum, M.S. and Eimstad, R. (1987a) 'Analysis of anaerobic biofilms', Environ. Technol. Lett., 8, 21-32.
Switzenbaum, M.S., Robins, J.P. and Hickey, R.F. (1987b) 'Immobilization of anaerobic biomass', In: G. Lettinga, A.J.B. Zehnder, J.T.C. Brotenhuisand L.W. Hulshoff Pol (eds), Granular anaerobic sludge microbiology and technology, Proc. GASMAT-Workshop, Lunteren, The Netherlands, October 25-27.
Tait, S.J. and Friedman, A.A. (1980) 'Anaerobic rotating biological contactor for carbonaceous wastewaters', JWPCF, 52, 2257-2269.
Trulear, M. and Characklis, W.H. (1982) 'Dynamics of biofilm processes', JWPCF, 54, 1288-1301.

Van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Schraa, G. and Zehnder,A.J.B. (1987) 'The role of bacterial cell wall hydrophobicity in adhesion', Appl. Environ. Microbiol., 53, 1893.

Veiga, M.C. (1989) 'Tratamiento por digestion anaerobia de los efluentes del procesado industrial de tunidos', Ph.D. dissertation, University of Santiago de Compostela, Spain.

Veiga, M.C., Mendez, R. and Lema, J.M. (1990) 'Anaerobic treatment of wastewater from a tuna processing industry in an anaerobic filter', Proc. Industrial Waste Water Treatment and Disposal, Nicosia, Cyprus, November 20-21.

Veiga, M.C., Jain, M.K., Hollingsworth, R., Pankratz, S. and J.G. Zeikus (1991) 'Characterization of exopolymer from methanogens'. Proc. Annual ASM Meeting, Dallas, TX, USA, May.

Veiga, M.C., Mendez, R. and Lema, J.M. (1992a). 'Treatment of tuna processing wastewaters in laboratory and pilot-scale DSFF anaerobic reactors', Proc. Purdue Industrial Waste Conference, West Lafayette, IN, pp. 447-453.

Veiga, M.C., Jain, M.K., Hollingsworth, R., Pankratz, S. and Zeikus, J.G.(1992b) 'Exopolymers isolated from biomethanation granules and from methanogens', (in preparation).

Verrier, D., Mortier, B., Dubourguier, H.C. and Albagnac, G. (1988) 'Adhesion of anaerobic bacteria to inert supports and development of methanogenic biofilms', Proc. of the 5th Int. Symp. on Anaerobic Digestion, Bologna, Italy.

Weiland, P. and Rozzi, A. (1991) 'The start-up, operation and monitoring of high-rate anaerobic treatment systems: Discusser's report', Wat. Sci.Technol., 24, 257-277.

Wilkie, A. and Colleran, E. (1984) 'Start-up of anaerobic filters containing different support materials using pig slurry supernatant', Biotechnol. Lett., 6, 735-740.

Wilkinson (1958) 'The extracellular polysaccharide of bacteria', Bacteriol Rev., 22, 46-73. Young, J.C. and Danhab, M.F. (1982) Operational characteristics of anaerobic packed-bed reactors. Biotechnol. Bioeng. Symp. No 13, p. 303, John Wiley and Sons, Inc., New York,USA.

Young, J.C. and Danhab, M.F. (1983) 'Effect of media design on the performance of fixed-bed anaerobic reactors', Wat. Sci. Technol., 15, 369-383.

Zeeuw, W. de, Hulshoff Pol, L.H., Boon, J. and Lettinga, G. (1982) 'Use of Factor 420 analysis in anaerobic digestion', In: Highes *et al.* (eds.) Anaerobic Digestion 1981, Elsevier Biomedical Press, Amsterdam, The Netherlands.

ANAEROBIC BIOFILMS IN FLUIDIZED BED REACTORS

H.-J. JÖRDENING
Institute for Agricultural Technology and Sugar Industry
Langer Kamp 5
3300 Braunschweig
Germany

1. Introduction

The anaerobic waste water treatment in fluidized bed reactors (FBR) has been studied in the last 15 years by many working groups all over the world (Switzenbaum (1982), Heijnen et al. (1985)). The main advantages of this process compared to other anaerobic processes are listed in Table 1.

Table 1: Comparison between FBR and other anaerobic technologies

Process		Contact	UASB	FBR
Height/diameter	m/m	1:1	1:1	4(7):1
Hydr. Retention Time	(d)	1-3	1-3	<<1
Superficial veloc.	(m/h)	1-5	0.5-1.5	8-50
Volatile susp. solids	(kg/m^3)	5-10	5-150	20-40
Volumetric loading	($kg/(m^3*d)$	5-12	10-20	50-240
Problems with solids		yes	yes	no

Despite of these advantages the number of installed technical plants is now still very low (Totzke (1988)). The reason for this are some problems, mainly with the time of development (Switzenbaum et al. (1980)) and the durability of anaerobic biofilms.

One of the most important factors are the chemical and physical properties of the carrier which is used as support material. there are many studies concerning several inorganic and organic carriers (Jördening, 1987). With respect to the full scale treatment the number of carriers is still low.

In this study results of laboratory investigations with fluidized bed reactors will be shown. The main interest is given to the development of biofilm with different inocula and support media.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 435–442.

2. Materials and Methods

The geometry of the reactor type, which was used for these studies is shown in Table 2.

Table 2: Reactors for laboratory and pilot scale studies

Height	m	1.6
Diameter	m	0.08
Total volume	m^3	$6.6*10^{-3}$
Working volume	m^3	$3.0*10^{-3}$
Packed bed vol.	m^3	$2.0*10^{-3}$
Expansion	-	1.5

Two carriers were studied as support material for fluidized bed reactors. Sand was used because of its low price and availability.

The foam glass (Poraver)[1)] was used because of its high porosity and its low density, which reduce the pressure loss in the fluidized bed. Foam glass is used by the construction industries as an isolation material. Therefore it is relatively cheap compared to other porous carrier. The properties of both materials are shown in Table 3 and 4. The fluid characteristics (eq. 1) could be calculated via a correlation developed by Richardson-Zaki (Couderc (1985)):

$$\ln v_S = \ln v_{ST} + n * \ln \varepsilon \qquad \text{(eq. 1)}$$

where v_S is the superficial fluid velocity, v_{ST} the bioparticle terminal settling velocity, ε the bed porosity and n a dimensionless expansion factor.

The power requirement per cubic meter expanded bed (P_w) for a 50 % expanded bed was measured as height difference (Δ H) between the water level of the reactor and a corresponding pipe and calculated with (eq. 2):

$$P_w = \Delta H * g * Q_L * \rho_f \qquad \text{(eq. 2)}$$

where Q_L is the volumetric flow rate and ρ_f the densitiy of the expanded bed.

Table 3: Fluid characteristics for sand and foam glass

Carrier		foam glass	sand
d	10^{-3}m	0.25-0.4	0.25-0.4
ρ_f	kg/m^3	1571	2550
ε	-	0.769	0.377
v_S	m/h	5.78	12.1
v_{ST}	m/h	105	171
n	-	12.3	3.03
a	m^2/m^3	$7*10^6$	$10*10^3$

The substrate contained acetate, propionate and butyrate (1:1:0.2) and a mineral solution (Brune

et al. (1982)). Calcium (1-2.7 kg/m^3) was added both to simulate a real sugar factory waste water and to get an pH of 6.

The COD (Chemical Oxygen Demand) in the reactor-effluent was kept on 0.1 kg/m^3, the temperature was set on 310 K, the redox potential at 260 mV measured with an Ag/AgCl-(3-M KCl) electrode.

Table 4: Energy requirement for fluidization

Carrier		foam glass	sand
V_L	m/h	0.0038	0.0041
Q_L	10^{-5}*m^3/s	1.94	2.08
ρ	kg/m^3	1084	1654
ΔH	m	0.034	0.195
P_w	W/m^3	4.67	46.4

3. Results

3.1. SAND AS CARRIER

Our first studies were carried out with sand as carrier. The start up of such a reactor is shown in Figure 1.

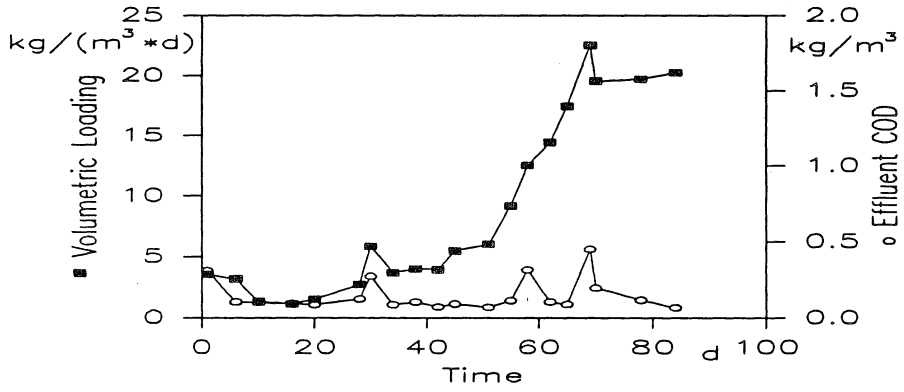


Figure 1: Start up of a fluidized bed reactor

The increase in performance respectively the development of biofilm is satisfactory, compared to the very long periods (up to nine month), which were discribed by Switzenbaum et al. (1980). But with respect to the boundary conditions which are given in a seasonal plant like a sugar factory, the time is to long, too.

A qualitative correlation can be seen between the development of biofilm and the precipitation of lime scale (Figure 3 and 4). Here, it can be assumed, that the two negatively charged surfaces of sand and of the bacteria will be crosslinked by the positively charged calcium. A further effect

may result by a growth of the specific surface/volume-ratio. A mathematical correlation between the precipitation of lime scale and the growth of the biofilm couldn't be found.

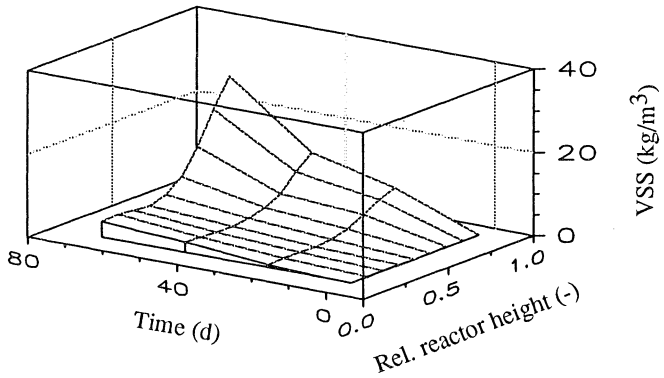


Figure 2: Biofilm development in the FBR

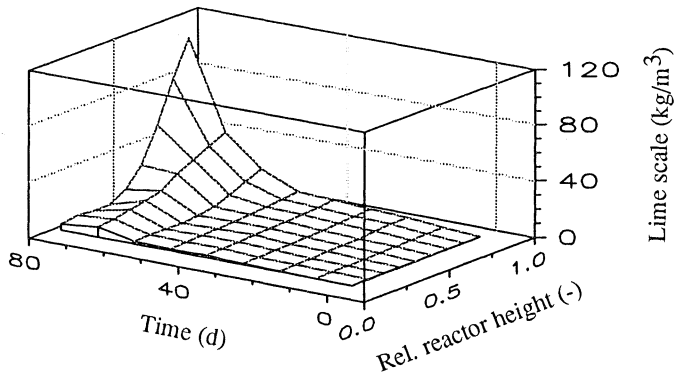


Figure 3: Lime scale precipitation in the FBR

Further studies were made to find out the behavior of anaerobic bacteria with respect to longer periods of standstill. For this reason the reactor was restarted after a standstill-period of 100 days. To get quickly more information about the reactor performance during this second working period the performance measurement were made by a kinetic method, which is described in Jördening et al. (1988).

The calculated kinetic data are shown in Table 5. As it can be seen, only a very short time (20 days) is necessary for restarting the reactor.

Table 5: Kinetic data to the restart of a FBR

Day after restart		2	5	15	18	25
k_M	kg/m3	0.057	0.062	0.072	0.059	0.063
V_{max}	kg/(m3*d)	10	10	17	21	28

Although the evaluated k_M's are only apparent values, they agree very well with those, determined by Gujer et al. (1983) for *Methanothrix soehngenii* (0.030 kg/m^3). Scanning electron micrographs showed a predominance of bacteria of this type against other methanogenic bacteria (like *M.brevibacter* and *M.spirillum*).

A screening of removed bacteria in several anaerobic fluid media showed, that even fermentative bacteria like Clostridium sp. and lactate producing bacteria are on the carrier (Wollersheim et al. (1988)). These organisms cannot utilize organic acids and may grow on lytic products of death bacteria.

3.2. FOAM GLASS AS CARRIER

Foam glass has a 1000 times bigger surface compared to sand but nearly the same chemical structure. From that it was interesting to investigate this factor concerning the growth of biofilm and performance of an anaerobic FBR.

Therefore three reactors were started with foam glass as carrier and different inoculation procedures. The reactor F-G 1 was started with anaerobic biomass from a contact reactor (the same as for the sand reactor), while the reactors F-G 2 and F-G 3 were inoculated with on foam glass immobilized biomass in different concentrations.

The reactor COD-concentration were kept on a level of 0.1 - 0.3 kg/m^3. In Figure 4 and 5 the increase of performance and the biofilm development during the start up period are shown for the three inoculation procedures. They are compared with the results obtained with the sand (see chapter 3.1).

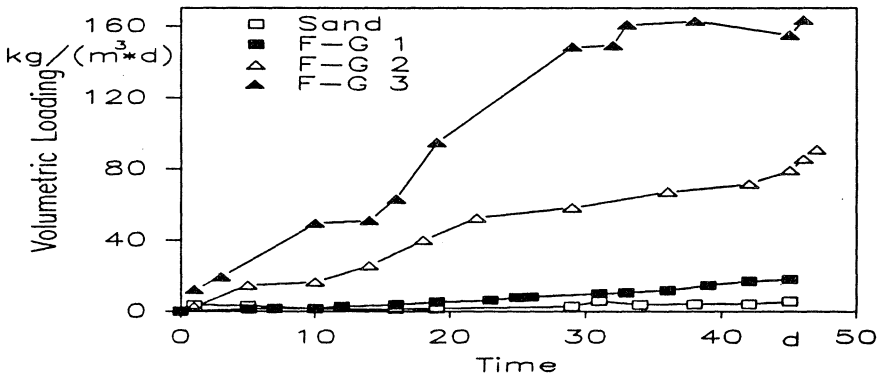


Figure 4: Performace during start up for differnt carrier

Both graphics show that foam glass provides much better conditions for microbial growth. The reason lies in the porous surface, which enables growth without big shear stress. This assumption will be supported by the observation, that the surface of sand is covered in the beginning mainly in cavities (Weise et al. (1978), Jördening (1987)).

For foam glass it seemed necessary to calculate an effectiveness factor because of the high lime scale and biomass contents on the carrier. For this calculations equations 3-5 (Shieh et al. (1986)) were used.

$$r = (r_p^3 - r_m^3) / 3r_p^2 \qquad \text{(eq. 3)}$$

$$\Theta_m = r * \mathrm{sqr}(\rho * k / (D * S)) \qquad \text{(eq. 4)}$$

$$\eta = 1.1302 * \Theta_m^{-0.9} \qquad \text{(eq. 5)}$$

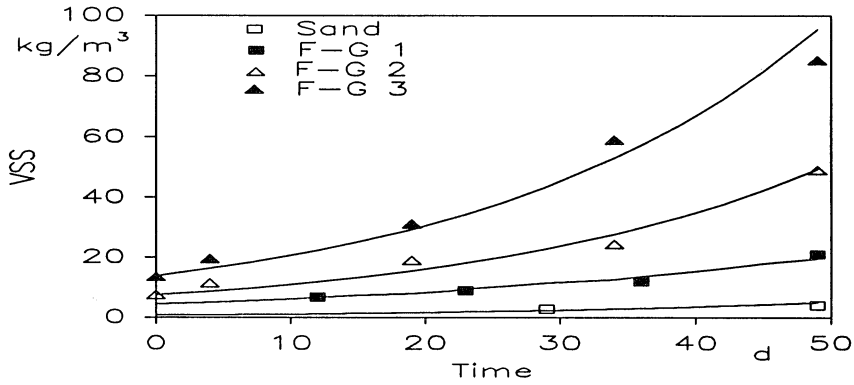


Figure 5: Biofilm development on different carrier

It was assumed, that foam glass has a smooth surface and a spherical shape. The kinetics were assumed to be of 0 order. This can be done because the K_m for acetate for Methanothrix is in the range of 0.030 kg/m^3 and the reactor value varied from 0.08-0.13 kg/m^3 .

With equation 1 and 2 the maximum value for the Thiele modulus was calculated to be 0.15.

This value is ten times lower then the one which would reduce the effectiveness factor (eq. 5). With respect to the irregular shape, which provides a larger surface than assumed, the real value of the Thiele modulus should be less then the calculated one.

The initial inoculum concentrations were compared with the calculated initial adhered biomass concentrations which can be derived by logarithmic regression of biofilm growth (lines in Figure 5).

From this it can be concluded that the start up phase can be shortened by using immobilized inoculum (Table 6).

There are two factors which influence the initial biofilm development. First the adhesion by bacteria seems to be better, if immobilized inoculum is used. The calculated higher values for the initial concentration (compared to the real added concentration) may be the result of the normal removing of excess biofilm, which quickly covers the new surface.

Table 6: Comparison of different inocula

Carrier		Sand	F-G 1	F-G 2	F-G 3
Initial conc.					
$VSS_{measured}$	(kg/m^3)	5.3	20	11	5.3
$VSS_{calculated}$	(kg/m^3)	0.78	5.9	13.9	7.4
μ_{max}	(d^{-1})	0.038	0.030	0.039	0.039
Start up time	(d)	115	146	112	112

The second factor is the higher growth rate (25 %) which even may be caused by the use of bacteria which were selected in a former reactor.

4. Summary and conclusions

The experiments showed that the choice of the carrier is of great importance for both the biofilm formation velocity and the attainable biomass concentration.

As shown for sand, calcium influenced the biofilm formation. It can be assumed that lime scale provides a secondary (positively charged) surface, which is easier to colonize by bacteria. A problem can arise from the growing diameter of the particles and the increase of the bed-density. This problem can be solved if sometimes a part of the carrier is removed and replaced by uncovered new carrier.

Immobilized biomass as inoculum gave much better results then suspended biomass. Here it should be tested, whether removing of biomass from the carrier and use of this biomass as inoculum can give a further increase in biofilm formation.

Even for high biomass (up to 85 kg/m^3) and lime scale (up to 200 kg/m^3) concentrations no decrease in the degradation rate by diffusion limitation could be calculated.

5. Nomenclature

a	Specific surface (m^2/m^3)
d	Diameter (m)
D	Diffusion coefficient (m^2/sec)
g	Gravitational acceleration
H	Height (m)
k_M	Reaction constant (kg/m^3)
n	Expansion factor (-)
P_W	Power
Q_L	Volumetric flow
r	Biofilm thickness (m)
r_p	Bioparticle radius (m)
r_m	Carrier radius (m)
S	Substrate concentration (kg/m^3)
v_S	Superficial upflow velocity (m/s)

v_{ST}	Terminal particle settling velocity (m/s)
V	Volume (m^3)
V_{max}	Max. reaction rate ($kg/(m^3*d)$)
VSS	Volatile suspensed solids (kg/m^3)

Greek letters

ε	Void fraction
η	Effectiveness factor
μ	Specific growth rate (d^{-1})
Θ	Thiele modulus
ρ	Density (kg/m^3)
ρ_f	Fluidized bed density (kg/m^3)

6. References

Brune, G., Schoberth, S.M. and Sahm, H. (1982) 'Anaerobic treatment of an industrial wastewater containing acetic acid, furfural and sulphite' Process Biochemistry 17, 20-35

Couderc, J.-P. (1985) 'Incipient fluidization and particulate systems' in Davidson, J.F., Clift, R. and Harrison, D. (eds.), Fluidization, Academic Press, London, 1-46

Gujer, W. and Zehnder, A.J.B. (1983) 'Conversion processes in anaerobic digestion' Wat. Sci. Tech. 15, 127-167

Heijnen, J.J, Mulder, A., Enger, W. and Hoeks, F. (1986) 'Review on the application of anaerobic fluidized bed reactors in waste-water treatment' EWPCA Conference on anaerobic waste water treatment

Jördening (1987) 'Untersuchungen an Hochleistungsreaktoren zum anaeroben Abbau von calciumhaltigen Abwässern' Doctoral thesis

Jördening, H.-J., Pellegrini, A. and Buchholz, K. (1988) 'Der Einsatz von Fließbettreaktoren zur anaeroben Abwasserreinigung von Zuckerfabriksabwässern' Zuckerind. 113, 1033-1039

Jördening, H.-J., Pellegrini, A., Brei, M. and Janssen, W. (1991) 'Optimization and upscaling of a fluidized bed system for anaerobic waste water purification' Zuckerind. 116, 1047-1052

Shieh, W.K. and Keenan, J.D. (1986) 'Fluidized bed Biofilm reactor for wastewater treatment' Advances in biochemical engineering/Biotechnology 33, 131-169

Switzenbaum, M.S. (1982) 'Biological filters' J. WPCF 50, 592-599

Switzenbaum, M.S. and Jewell, W.J. (1980) 'Anaerobic attached-film expanded-bed reactor treatment' J. WPCF 52, 1953-1965

Totzke, D.E. (1988) 'Anaerobic treatment technology - 1988 Update' Food processing waste conference, Atlanta

Weise, W. and Rheinheimer, G. (1978) 'Scanning electron microscopy and epifluorescence of bacterial colonization of marine sand sediments' Microbial Ecology 4, 115-122

Wollersheim, R., Jördening, H.-J., and Diekmann, H. 'Veränderung der Biomassezusammensetzung bei der Inbetriebnahme eines anaeroben Wirbelschichtreaktors' gwf-Wasser/Abwasser 129, 127-132

BIOFILM GROWTH IN AN ANAEROBIC FLUIDIZED BED REACTOR

V. DIEZ, F. FDZ-POLANCO, P.A. GARCIA.
Dept. of Chemical Engineering
University of Valladolid
47011 Valladolid.
Spain.

1. Introduction

The fluidized bed technology can offer many advantages related with the use of small particles of support material for the accumulation of the anaerobic bacteria and with the recirculation and turbulence characteristic of the system (Iza, 1.991):

a) very large surface area for biomass attachment,
b) biofilm developed on the carrier material cannot be easily washed out from the reactor.
c) reduction of substrate concentration and of the shock effect of toxicant spikes,
d) high mass transfer properties, allowing the treatment of low strength wastes.

The biofilm growth can modify the physical properties of the bioparticles. When the biofilm covers the particles, the diameter, shape and size of the bioparticles vary, modifying the hydraulic behaviour of the bed.

2. Materials and Methods

The reactor is a lab-pilot scale AFB of 19.3 cm diameter and 188 cm height. The column has 6 sampling ports uniformly located, numbered from the bottom. The process flow sheet is shown in a previous communication (Fdz-Polanco *et al.*, 1.991).
The reactor was continuously fed with synthetic wastewater prepared by mixing tap water and a concentrated solution of acetic acid, alkali and the required nutrients for microorganisms growth. The concentrated synthetic wastewater composition is presented in table 1. The concentrated solution pH was approximately 4.5, sufficiently low to prevent the organic material degradation in the stock solution recipient.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 443–448.

CH_3COOH	150. (g/l)
NH_4HCO_3	9.3
$NH_4H_2PO_4$	1.0
$MgSO_4.7H_2O$	0.6
$CaCl_2$	0.044
NaOH	40

Table 1. Concentrated solution composition.

The reactor was filled with a clay (sepiolite 30/60 C) up to 100 cm height of fixed bed. The bed fluidization leads to the particles segregation at the different levels of the reactor. The average support material properties were:

particle diameter, d_p	0.046 cm
dry bed apparent density, ρ_d	0.56 g/cm^3
particle density, ρ_p	1.44 g/cm^3
sepiolite crystalline density, ρ_c	2.20 g/cm^3

Table 2. Average support material properties.

Every week bioparticles were sampled at the different levels of the reactor, and was determined, in accordance with Standard Methods (1.985), the volatile attached solids concentration, apparent density of the bed, and apparent density of the calcined bed. From this experimental values the relative volume or the relative thickness of the biofilm, the biofilm moisture, wet biofilm density and dry biofilm density were calculated. To determine these parameters it was accepted that the porosity of the bed at the fluidization onset was 0.41, slighting the presence of fixed solids in the biofilm and assuming for the relative thickness that the particle shape is spherical (Diez, 1.991).

3. Experimental conditions

The reactor was seeded with inoculum from an UASB reactor of a beet sugar factory. Operation temperature was 35 °C.
Fixing the hydraulic retention time, t_R, and the organic loading rate, B_v, it is possible to distinguish five operation periods characterized by the conditions shown in table 2.

Period	B_v (g O_2/l · d)	t_R (h)
1	2	18
2	5	11
3	10	7
4	21	4
5	35	5

Table 3. Operational conditions of the AFBR.

4. Results

4.1. Attached Volatile Solids Concentration (SV_{att})

The biofilm growth was monitored by the evolution of the attached volatile solids, despite of the limitations this parameter in the measurement of active microorganisms. Attached volatile solids concentration is affected, for example, by the presence of:

- no active microorganisms (dead microorganisms),
- organic extracelluar polymers forming the organic matrix,
- adsorbed soluble substances.

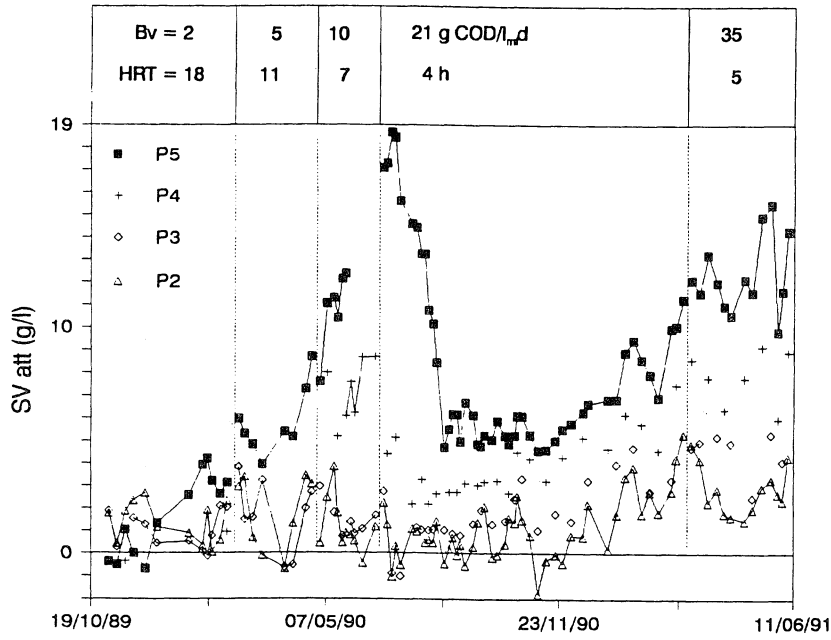


Fig. 1. Evolution of volatile solids at different levels of the reactor.

Figure 1 shows the temporal evolution of attached volatile solids concentration. The difference in the evolution of the particles located at upper and lower parts of the reactor can be clearly appreciated. At the top, the rate of biofilm growth is faster. On the other hand, the attached volatile solids concentration at the top increases when the organic loading rate increases, until the first organic over-load that destabilized the process.
At the lower levels, the microorganisms growth is slower, reaching low concentrations that remain practically constant when the organic loading rate increases.

4.2. Relative Biofilm Volume

Another way to control biofilm growth is derived from the relative biofilm volume, obtained comparing the volumes of the bed formed by bioparticles and the one formed by clean particles, and calculated from the equation:

$$\frac{V_f}{V_{bed}} = (1-\epsilon_p) - \frac{V_{550}}{V}\cdot(1-\epsilon_s)$$

where V is sample volume, V_{550} is volume after calcination, and ϵ is the porosity of the bed formed by the bioparticles (p), or by clean sepiolite (s). The constant porosity values determined in preliminary observations, were $\epsilon_p = 0.41$, and $\epsilon_s = 0.40$.
Accepting this values for the initial period, when the carrier material is poorly colonized, the equation leads to negative values of the biofilm volume. Figure 2 shows that the relative biofilm volume evolution is similar to the evolution of the attached biomass concentration. These results point to a clear segregation of the bed, similar to that exposed in the previous section.

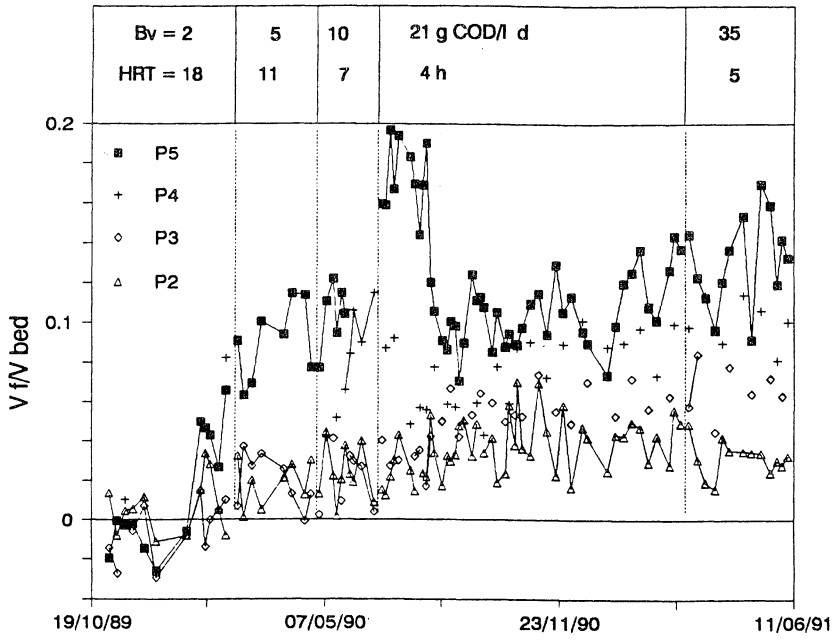


Fig. 2. Evolution of biofilm relative volume at different levels of the reactor.

4.3. Biofilm Moisture

The biofilm moisture is defined as the relation between the water mass in the biofilm and the total biofilm mass. If biofilm was formed only by volatile solids and water, being unaware of fixed solids in the biofilm, the biofilm moisture, P, can be calculated as:

$$P = \frac{W_f}{W_f + SV_{att}} \; ; \qquad W_f = \frac{(m - m_{105}) - SV^o \cdot V_{550} - (\varepsilon_p \cdot V + V_{550} \cdot (1 - \varepsilon_s) \cdot \chi_i) \cdot \rho_w}{V}$$

where W_f is the water mass in the biofilm by unit of bed volume, m is wet bed mass, m_{105} is dry bed mass, SV^o is sepiolite mass decrease due to its crystallization water, and χ is sepiolite internal porosity.
Although the biofilm moisture slightly varies with the biomass concentration, it is possible to establish a mean value, $P = 0.94$ ($\sigma = 0.04$). As it was expected, most of the biofilm was formed of water.

4.4. Wet Biofilm Density

The wet biofilm density, ρ_{bw}, can be determined by the equation:

$$\rho_{bw} = (SV_{att} + W_f) \cdot \frac{V}{V_f}$$

In figure 3 it can be observed that the range of the most frequent wet biofilm density is 1.00 - 1.05 g/cm^3, logically close to water density.

5. Conclusions

Our results point to a clear segregation of the bed. The behaviour of the particles located at the bottom could be related with the micro-scale shear forces generated by the distribution system and with the abrasion from particle-particle interaction.
The experimental results prove that the particle density decrease, due to the biofilm growth, is not the only reason for segregation. It was checked that the non-homogeneity of the support material, that is responsible of the initial segregation, remains during all the operation time.
In our experimental conditions the effect of mixing produced by the biofilm growth and biogas production is practically negligible, not modifying the initial stratification of the bed.

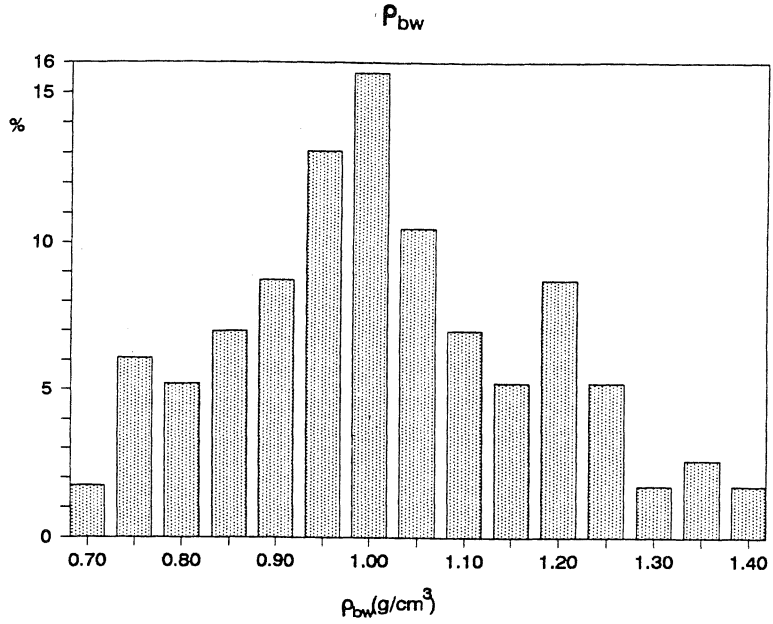


Fig. 3. Wet biofilm density.

Acknowledgments

The authors gratefully acknowledge support of this study by CICYT (Proyecto BT 11/87) and FPI grant of Spanish Ministry of Education.

6. References

Diez, V., 1.991. 'Evolución y comportamiento hidrodinámico de un biorreactor anaerobio de lecho fluidizado'. Tesis Doctoral. Universidad de Valladolid. Valladolid

Fdz-Polanco, F., García, P.A. and Diez, V., 1.991. 'Biofilm evolution in an anaerobic fluidized bed reactor (AFBR)'. International Symposium Environmental Biotechnology. Part I, 291-294. Oostende, Belgium.

Hickey, R.F., 1.990. 'Principles for use of adsorbent carriers in fluidized bed reactors'. In: Biological Fluidized Beds for Water and Wastewater Treatment: A User's Forum. Ann Arbor, Michigan.

Iza, J., 1.991. 'Fluidized bed reactors'. Wat. Sci. Tech. 24 (8), 109-132.

Jewell, W.J., 1.985. 'The development of anaerobic wastewater treatment'. Proc. Seminar/workshop Anaerobic Treatment of Sewage, Switzenbaum, M.S., Editor, Amherst, Massachusetts.

Shieh, W.K. and Keenan, J.D., 1.986. 'Fluidized bed biofilm reactor for wastewater Treatment'. Advances in Biochem. Eng./Biotech. 33, 132-169.

Standard Methods for Examination of Water and Wastewater, 1.985, 16th ed., APHA, AWWA, WPCF, Washington.

INTEGRATED NITRIFICATION AND DENITRIFICATION WITH IMMOBILIZED MICROORGANISMS

V.A. SANTOS, J. TRAMPER, R.H. WIJFFELS
Wageningen Agricultural University,
Food and Bioprocess Engineering Group
P.O.Box 8129
6700 EV Wageningen
The Netherlands

Introduction

The discharge of nitrogenous compounds in the environment constitutes a major pollution problem, seriously enhancing acid rains formation, soil acidification, nitrate leakage to ground water, excessive oxygen demand and euthrophication.
Nitrogen compounds can be removed within wastewater treatment systems by nitrification followed by denitrification. In this multistep process, nitrification (oxidation of ammonia to nitrate via nitrite) is in particular problematic due to the slow growth rates of the nitrifying bacteria, which often leads to the wash-out of the cells from the conventional treatment tanks. Furthermore, the long generation times and the low affinity for oxygen of these bacteria make them very inefficient when in competition with heterotrophic microorganisms. Thus, long retention times and large volume reactors are required in treatment plants.

Nitrification can efficiently be accomplished in air-lift loop reactors with immobilized cells since a much higher concentration of biomass is attainable in such systems (Tramper and Grootjen (1986), Wijffels and Tramper (1989), Wijffels et al (1990)). As the cells are retained in the reactor, it is possible to operate under high dilution rates, which is particularly advantageous in wastewater treament. In such immobilized systems, the process is regulated to a great extent by the substrate transfer rates, particularly oxygen, thus being much less dependent of the kinetic properties of the microorganisms and bacterial growth rates (de Gooijer et al (1990)). Consequently, the overall volumetric productivity of such bioreactor systems can be largely enhanced.

The effluent of those reactors still contains nitrate, which needs to be removed as well. For that, it is possible to introduce an anaerobic (biological) denitrification step in which nitrate is converted into nitrogen gas at the expense of the oxidation of organic compounds. This denitrifying step is in principle simple, but in practice difficult to combine with nitrification in one process, since the oxidation-reduction potential acts in opposite ways in these biochemical reactions. Different environments are thus required by the two microorganisms to perform efficiently both processes.

In conventional purification systems organic compounds are first oxidized and in a second step nitrification can take place. To integrate denitrification in such a process, an anaerobic stage can be introduced as a first or last step. If it is introduced as a first step, part of the

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 449–453.

effluent of the nitrifying reactor has to be recycled. The result is, that part of the produced nitrate will not be converted. If it is introduced as a last step, a part of the influent has to be by-passed to the denitrification reactor in order to introduce organic compounds. As a result some nitrogen is discharged as ammonia with the effluent. In none of these situations a complete nitrogen removal is achieved.

In this paper, the possibilities of complete nitrification and denitrification within a single reactor system are evaluated.

Co-immobilized microorganisms

In theory, integrated nitrification and denitrification can be achieved within a gel particle with a co-immobilized mixed-culture system. Nitrification is an aerobic process which readily occurs in gel beads with either pure or mixed immobilized cultures of Nitrosomonas and Nitrobacter, as previously shown (Wijffels and Tramper (1989), Wijffels et al (1990), Wijffels et al (1991)). It was also demonstrated that in such immobilized-cell systems diffusion limitation phenomena are likely to occur. Oxygen diffuses into the gel particles and is used by the immobilized nitrifying organisms. Since the cell density is high in the gel beads, oxygen is consumed at a fast rate and oxygen depletion in the center of the bead will occur due to diffusion limitation. As the cell growth rate is strongly dependent of the oxygen concentration, a biomass density gradient then will develop. The nitrifying bacteria will tend to concentrate in a biofilm just under the surface of the gel particle, leaving the anoxic central part completely unused. Thus, denitrification may occur in the bead core if suitable denitrifying bacteria are present and if organic substrate is available. The metabolic product of metabolism of one microorganism is then the substrate for the second one. The situation is that of a habitat segregation of the two microorganisms under aerobic conditions (see Figure 1).

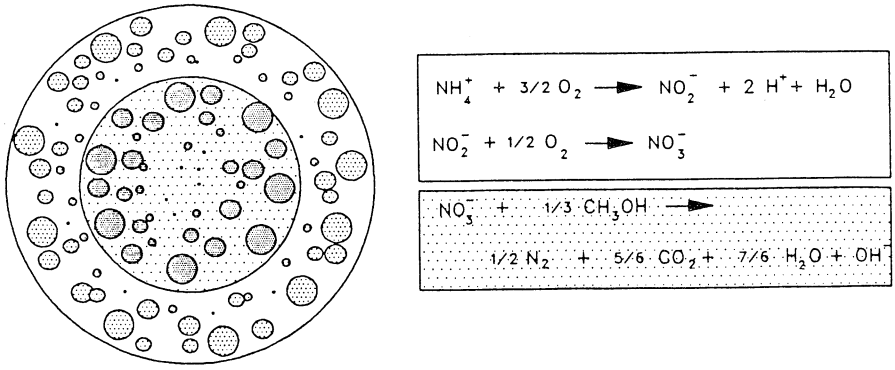


Figure 1. Schematic representation of the "Magic Ball" concept. Product formation in a co-immobilized mixed culture system. Dotted area: denitrification; plain area; nitrification .

Both kind of microorganims have similar ranges of optimal pH and temperature (Nilsson et al (1980), Kokufuta et al (1986), Wijffels, Schukking and Tramper (1990), van Ginkel et al (1983), Tramper and de Man (1986)) which means that it is in principle possible to operate in culture conditions close to their optima.

However, a co-immobilized mixed culture system, with bacterial segregation, is spontaneously established only when the oxygen demands of the component microorganisms are significantly different (Kurosawa and Tanaka (1990)), which is not the case with nitrifying and denitrifying bacteria. In fact, either Paracoccus denitrificans or Pseudomonas denitrificans, abundant denitrifying organisms, are facultative anaerobic bacteria that will compete for oxygen uptake with Nitrosomomas europaea under aerobic conditions. Furthermore, these denitriying microorganisms have growth rates of about one order magnitude higher than nitrifying bacteria, which certainly enlarges the problem.

It is thus desirable to develop a method to segregate nitrifiers and denitrifiers efficiently in a gel bead, providing a suitable environment for each microorganism. An attractive possibility is to separate both microorganisms physically, either by coating pre-formed gel beads or by double-bead formation using polyanionic and polycationic gels.

At present, we are investigating this direction and preliminary results were successful with respect to the coating of κ-carrageenan beads (in which denitrifying cells were immobilized) with a κ-carrageenan layer containing nitrifying bacteria. We call this the "Magic Ball" concept (see Figure 1).
Currently, we are working on the characterization and performance evaluation of such a system with immobilized Nitrosomonas europaea and Pseudomonas denitrificans.

Multiple gas-lift loop reactor

Alternatively, complete nitrification and denitrification could be strived for in a series of gas-lift compartments in one reactor. In the first aerobic compartment nitrification with immobilized cells should be executed. As the retention time in this part can be very short (10-15 min) and the turbulence very high, heterotrophic organisms will not be retained in this part and will not compete with the nitrifying bacteria. In the second step heterotrophic denitrifying bacteria are immobilized. Simultaneous nitrate reduction and oxidation of organic compounds will take place under anoxic conditions, since nitrogen gas is sparged instead of air. The transfer from one compartment to the other is simply done by overflow.

A suitable reactor for this process is the multiple gas-lift loop reactor, which is currently being developed at the Wageningen Agricultural University (Bakker, de Gooijer and Tramper (1992), Dutch patent (1989)).

In itself, a series of gas-lift loop reactors is built in one vessel (see Figure 2). The succeeding gas-lift reactors are concentrically placed and divided each in a riser and a downcomer section by a circular baffle. Fresh medium is supplied to the central air-lift reactor which overflows into the downcomer of the next compartment (sparged with nitrogen gas). Here it will be mixed with the down-flowing stream. The number of compartments in the series can be chosen as desired.

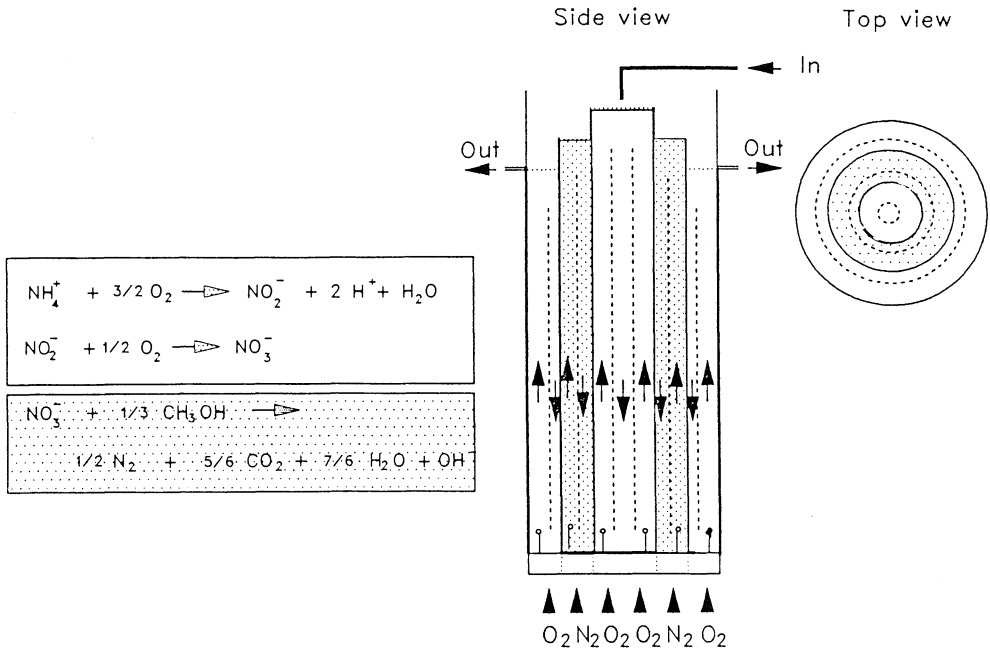


Figure 2. Integrated nitrification and denitrification in a Multiple Gas-lift Loop Reactor. Dotted area: denitrification; plain area: nitrification.

Conclusions

Both nitrification and denitrification with several immobilized microorganisms in gel beads, have been described in the literature. Each individual system is relatively well characterized concerning to its kinetic properties, physical/chemical characteristics and dynamic behaviour. Models (de Gooijer et al (1991), Wijffels et al (1991) were developed in order to understand the complex interactions inside immobilized cell particles, aiming to describe and predict the system's behaviour. However, this knowledge has been focused mainly on the single processes themselves but not much has been done on the integrated system.
Both strategies described here show promising potentialities. Additionally, in both cases there is the possibility of adding an organic matter removal step for application in wastewater treatment.

Considerable research and effort is necessary to assess the feasibility of such systems and to quantitatively compare them with conventional processes. If such a configuration shows to be feasible, then much could be done in view of more efficient ways of water treatment with important cost reductions.

References

Bakker, W.A.M., de Gooijer, C.D., Tramper, J. (1992) 'Physical characterization of the multiple air-lift loop reactor (MAL)', in Proceedings of the 4th Netherlands Biotechnology Congress, A.A. Balkema (publ.), Rotterdam.

Dutch patent nr: NL 8901649 (1989), 20pp.

van Ginkel, C. *et al.* (1983) 'Characterization of *Nitrosomonas europaea* immobilized in calcium alginate', Enzyme Microb. Technol., 5, 297-307.

de Gooijer, C.D., Wijffels, R.H. and Tramper, J. (1991) 'Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in carrageenan : Part 1- Dynamic modelling', Biotech. Bioeng., 38, 224-231.

Kokufuta, E., Shimoshashi, M. and Nakamura I. (1986) 'Continuous column denitrification using polyelectrolyte complex-entrapped *Paracoccus denitrificans* cells', J. Ferment. Technol., 65, 359-361.

Kurosawa, H. and Tanaka, H. (1990) 'Advances in immobilized cell culture : development of co-immobilized mixed culture system of aerobic and anaerobic microorganisms', Process Biochem. Int,, December, 189-196.

Nilsson, I. *et al.* (1980) 'Denitrification of water using immobilized *Pseudomonas denitrificans* cell', Europ. J. Appl. Microb. Biotech., 10, 261-274.

Tramper, J. and Grootjen, D.R. (1986) 'Operating performance of *Nitrobacter agilis* immobilized in carrageenan', Enzyme Microb. Technol., 8, 477-480.

Wijffels, R. and Tramper, J. (1989) 'Performance of growing *Nitrosomonas europaea* cells immobilizaed in *k*-carrageenan', Appl. Micron. Biotech., 32, 108-112.

Wijffels, R.H. *et al.* (1990) 'Nitrification with immobilized bacteria in air-lift loop reactors : modelling and application', in C. Christiansen, M. Munck and J. Villadsen (eds.) Proceedings of the 5th European Congress on Biotechnology, Copenhagen, 1, 392-395.

Wijffels, R.H., Schuking, G. and Tramper, J. (1990) 'Characterization of a denitrifying bacterium immobilized in *k*-carrageenan', Appl. Microb.Biotech., 34, 399-403.

Wijffels, R.H., de Gooijer, C.D., Kortekaas, S. and Tramper, J. (1991) 'Growth and substrate consumption of *Nitrobacter agilis* cells immobilizaed in carrageenan : Part 2-Model evaluation', Biotech. Bioeng., 38, 232-240.

BIOFILMS IN MEMBRANE BIOREACTORS FOR WASTE GAS TREATMENT

M.W. REIJ, G.T.H. VOSKUILEN and S. HARTMANS
Division of Industrial Microbiology
Department of Food Science
Agricultural University
P.O. Box 8129, 6700 EV Wageningen, The Netherlands

1. Introduction

Reduction of air pollution is becoming more and more important.
One of the promising methods to remove organic compounds from waste gas is to treat the gas in a bioreactor. In such a bioreactor the contaminants are transferred from the gas phase into the water phase and subsequently degraded by microorganisms.

2. Removal of poorly soluble organic compounds

A major problem in biological waste gas treatment is the removal of poorly soluble organic compounds. These compounds have a high air/water partition coefficient (m).

m is defined by $\frac{\text{concentration in gas phase} \quad [\text{mol/m}^3]}{\text{concentration in water phase} \quad [\text{mol/m}^3]}$

The partition coefficient strongly affects the flux (J) into the water phase, in which the microorganisms are present (Fig. 1).

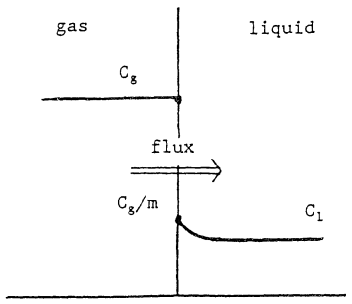


Fig. 1: Concentrations in gas and liquid phase of a poorly soluble compound

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 455–460.

$J = k * A * (\text{driving force}) = k * A * ((S_g / m) - S_l)$ [kg/s]

J	= mass flux	[kg/s]
k	= mass transport coefficient	[m/s]
A	= area	[m^2]
S_g	= substrate in gas phase	[kg/m^3]
m	= partition coefficient	[-]
S_l	= substrate in water phase	[kg/m^3]

A large m results in a small driving force and consequently a small flux. Since the constant m can not be manipulated the factor (k * A) has to as big as possible to maximize the removal efficiency of poorly soluble volatile compounds.

A possible solution is a membrane bioreactor in which a hydrophobic porous membrane is used to create a large interface between the gas- and water phase. As the membrane material is hydrophobic, the pores are filled with gas (Fig. 2) allowing high diffusion rates through the pores.

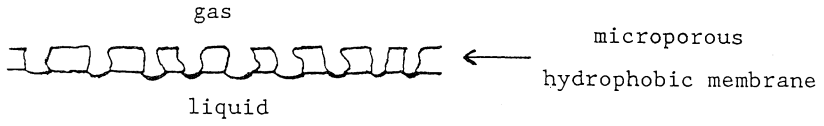


Fig. 2: Schematic drawing of a hydrophobic porous membrane

In hollow fibre membrane modules very high values for the specific area (a) are feasible (up till 10,000 m^2/m^3).

The mass transfer coefficient k is important as well. By inserting a membrane between gas and liquid an additional resistance is introduced. The resistance of the membrane, however, is very small corresponding to the resistance of a stagnant layer of gas of 1.3 mm. The mass transfer coefficient of the membrane was proved to be 0.01 m/s for ethanol transport.

A membrane reactor seems to be a feasible possibility to create a large contact area between liquid and gas. This interface can be created without the energy costs that are required with a number of other reactor types.

3. Biofilm formation

In the membrane bioreactor microorganisms are expected to form a biofilm on the liquid side of the membrane. Several experiments have been performed to study biofilm formation in small scale membrane bioreactors.

The 40 cm^2 membrane separates the reactor into two compartments. Liquid flows through one compartment and air containing volatile contaminants

flows through the gas compartment of the reactor.

Before inoculation the membrane reactor is sterilized. After sterilization the inoculum is circulated for 1 day to inoculate the surface. After inoculation the liquid flow is changed from circulation to once-through mode.

The biofilm that forms is supplied with oxygen and a carbon and energy source from the gas side, and with water, nitrogen and other minerals from the liquid side (Fig. 3).

Biofilm formation and substrate conversion was studied with the following combinations of substrates and microorganisms:

substrate	partition coefficient m (conc. gas / conc. liquid)	microorganism
dichloromethane	0.13	strain DM21
toluene	0.26	*Pseudomonas* GJ40

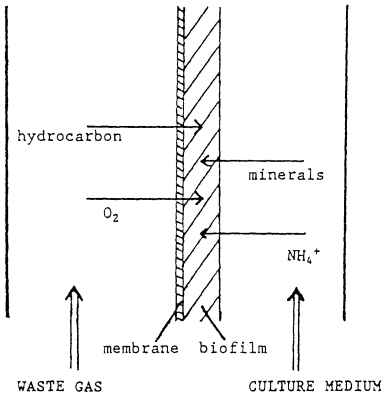


Fig. 3: Schematic view of fluxes into a biofilm supported by a membrane.

3.1. DICHLOROMETHANE REMOVAL

To study the removal efficiency of dichloromethane by strain DM21 a membrane bioreactor was inoculated with dichloromethane grown DM21 cells. The gasflow along the membrane was 100 ml/min containing 255 mg/m^3 dichloromethane.

After 1 day of inoculation the flow of mineral salts medium was switched to once-through mode and the dichloromethane conversion was followed in time (Fig. 4). Conversion was determined by measuring the dichloromethane concentrations in the in- and outgoing air.

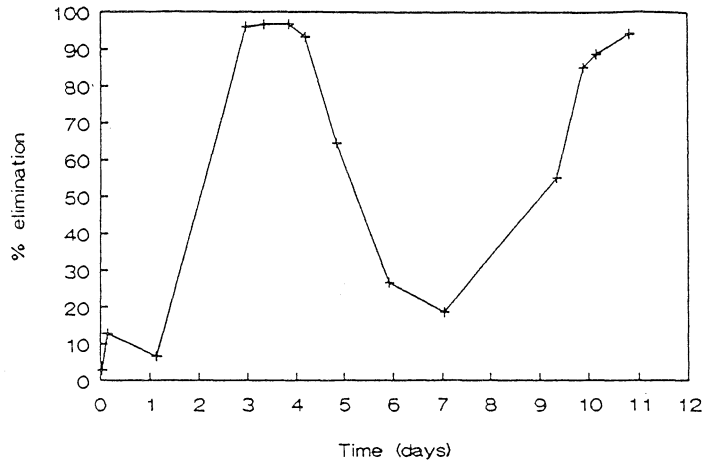


Fig. 4: Dichloromethane conversion by DM21 in a 40 cm^2 membrane bioreactor.

A drop in conversion was observed after 4 to 5 days. This coincided with a clearly visible biofilm being almost completely sheared off. After 7 days a new biofilm began to develop and conversion increased again.

We assume that the high conversion of dichloromethane during the first days of the experiment results in a pH gradient in the biofilm resulting in cell death and loss of biofilm structure.

3.2. TOLUENE REMOVAL

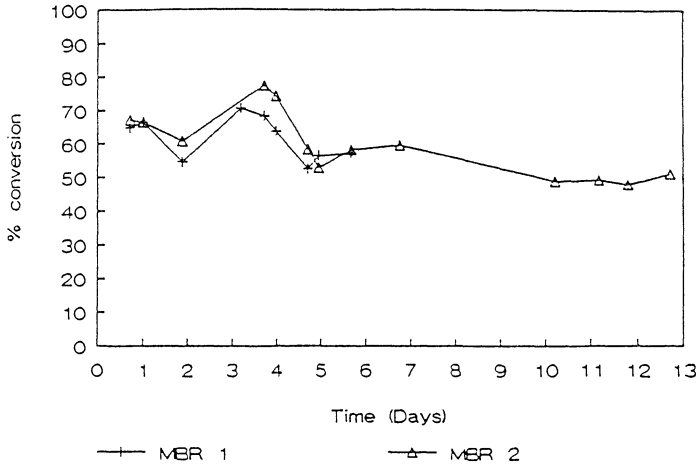


Fig. 5: Conversion of toluene by *Pseudomonas* GJ40 in two 40 cm^2 reactors

Biofilm formation and toluene degradation by *Pseudomonas* GJ40 was studied in a membrane bioreactor.

The toluene concentration in the gas phase was 190 mg/m^3 and the gas flow 100 ml/min. Conversion was measured in time and shown in Fig. 5. After 2 to 3 days biofilm formation could be visually observed.

Two reactors were dismantled after 6 respectively 13 days of operation and the biofilms were analyzed for protein content and maximum CO_2 production activity. The analysis was performed by cutting the membrane into 9 pieces. The pieces are numbered 1 to 9 from the air inlet to the air outlet (Fig. 6a and 6b).

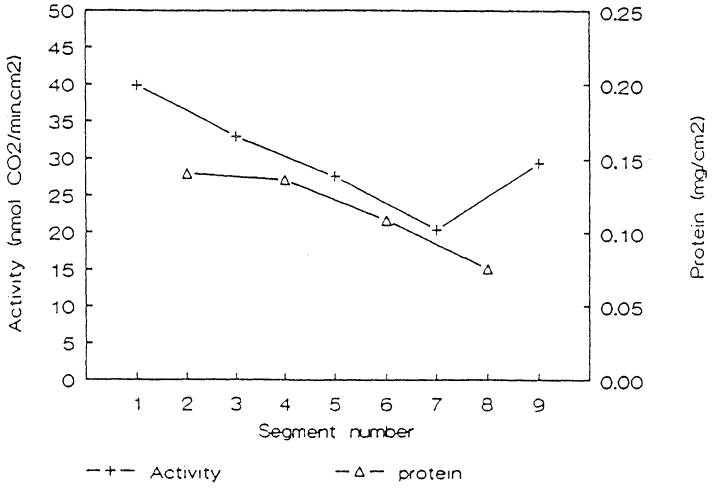


Fig. 6a: Protein content and activity of a 6 day old biofilm of GJ40.

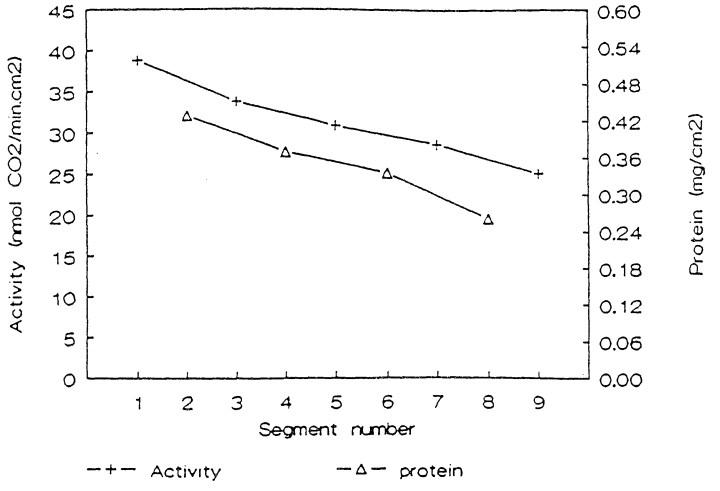


Fig. 6b: Protein content and activity of a 13 day old biofilm of GJ40.

In Fig. 6a and 6b it can be seen that the activity profile is similar for the two biofilms, ranging from 20 to 40 nmol CO_2/min.cm^2.

The protein content however differs considerably. The protein content of the 13 days old biofilm is about twice the content of the 6 days old biofilm. At day 13 the cells apparently are less active then at day 6. This might indicate that growth (at the membrane side) and cell death (at the liquid side of the biofilm) has occurred.

4. Conclusions

Initial experiments of the removal of toluene and dichloromethane have shown that degradation of organic compounds in a membrane bioreactor by biofilms is possible. Future experiments will concentrate on the degradation of poorly soluble compounds e.g. propene and long term operation and stability of the active biomass.

BIOFILMS IN PEAT BIOFILTERS

A. M. MARTIN
Department of Biochemistry
Memorial University of Newfoundland
St. John's, Newfoundland, Canada, A1B 3X9

1. Introduction

1.1 USE OF PEAT IN WASTE TREATMENT OPERATIONS

As a potential raw material for a variety of uses, peat has been recognized to possess a unique combination of chemical and physical properties, such as absorbency, adsorbency and deodorization, which could be employed in environmental protection applications (McLellan & Rock, 1986; Martin, 1991). Mueller (1972) discussed peat's potential in pollution abatement associated with its capacity to adsorb organic and inorganic matter and its properties as a filter material. The main mechanisms for the attachment of pollution to peat filters are shown in Figure 1.

Recently, the need to dispose of toxic wastes has generated renewed interest in the properties of peat that could be employed towards those aims. Treatment of gaseous effluents has also been highlighted as one of the possible applications for which peat properties are suitable.

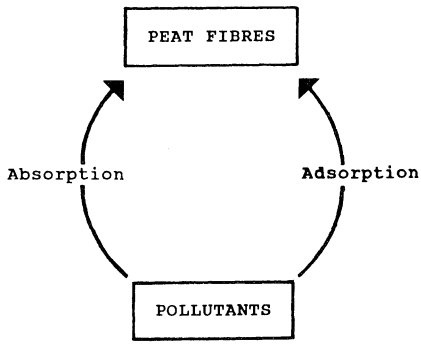


Figure 1. Main mechanisms for the attachment of pollutants to peat.

1.2 PEAT AS A FILTERING AGENT

In comparing peat with one of the most accepted media for filtration and wastewater treatments, *i.e.* activated carbon, it has been noted that the surface of the latter is, for the most part, nonpolar. This makes carbon a good adsorbent for organics, but the adsorption of inorganic electrolytes is more difficult (Netzer & Hughes, 1984). The major drawback attributed to the usage of activated carbon is its expense (McLellan & Rock, 1986). In addition to the high surface area (>200 m^2/g, 95 %

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 461–466.

porosity) of peat, and its adsorption properties, it has the advantage of supporting the formation of biofilms where microbial degradation can occur.

1.3 AIR AND GAS PURIFICATION

Mueller (1972) referred to the high carbon content of dried peat moss, its large surface area to weight ratio and its adsorptive properties. He also mentioned the potential of peat to adsorb odours, by virtue of its physical and chemical structure, in the same way that activated carbon is being used by industry. Pomeroy (1982) discussed the important role of microorganisms in the treatment of odorous air. At the beginning, efforts were confined to chemical and physical methods. Then, the importance of biological methods in pollution control was realized. Smith *et al.* (1973) showed the importance of microbial action in the capability of soil to degrade SO_2, H_2S, CH_3SH, CO, C_2H_2 and C_2H_4. Furusawa *et al.* (1984) used a packed bed of fibrous peat as a deodorizing material to remove H_2S from air in a laboratory-scale column. This removal was mostly due to biological oxidation by indigenous microorganisms in the peat. Wada *et al.* (1986) determined the characteristics of the H_2S oxidizing bacteria inhabiting a peat biofilter.

1.4 PEAT AS A SUPPORT FOR MICROORGANISMS

In addition to peat's chemical and physical characteristics, its biological properties should be taken into consideration when analysing its effect in pollution control. It has been long recognized that some of the properties of peat are appropriate for maintaining microorganisms in viable condition. Lochhead & Thexton (1947*a,b*) found in comparative tests of various powdered materials that peat was superior to other preparations for maintaining viable test bacteria. Jaouich (1975), in his studies on nitrate reduction in peat, reported the role of microorganisms in the denitrification process and the isolation of representative strains of denitrifying *Pseudomonas* species found in peat. Nichols & Boelter (1982) in their studies on the treatment of secondary sewage effluent with a peat-sand filter bed, found that microbial immobilization in the peat contributed to nitrogen and phosphorus removal.

This presentation reviews and discusses the potential of peat utilization in pollution control, with emphasis on the biodegradative processes that occur with the formation of a biofilm on the surface of the peat fibres. The characteristics of peat as a support for microbial populations will be highlighted. A new theory is presented, suggesting that peat could play the role of an active agent for biological degradation. This role could complement the present use of peat in waste treatment, which is mostly based on its physical and chemical properties. A schematic view of the potential of peat in the removal of pollutants is presented in Figure 2.

2. Materials and Methods

2.1 BIODEGRADATION SYSTEM

Although a bioprocessing module designed for liquid waste treatment has been utilized, this paper will deal with a biofilter unit packed with fibrous peat moss, employed for deodorizing gases (Furusawa *et al.*, 1984). For experiments utilizing gaseous pollutants, hydrogen sulphide gas is generated by the method shown in Figure 3, and initial tests have been conducted with *Thiobacillus* species, a facultative autotrophic bacterium able to degrade sulphur compounds. It is expected that, in the testing of specific effluents, other microbial strains, adapted for

continuous degradation of the selected contaminants, will be isolated from the biofilms in the peat filters. In the treatment of both liquid and gas effluents, a set of experiments, using standard procedures, need to be conducted without previous immobilization of microorganisms, to detect the presence in the peat of native biodegradative microbial populations and their enrichment when exposed to the effluent components.

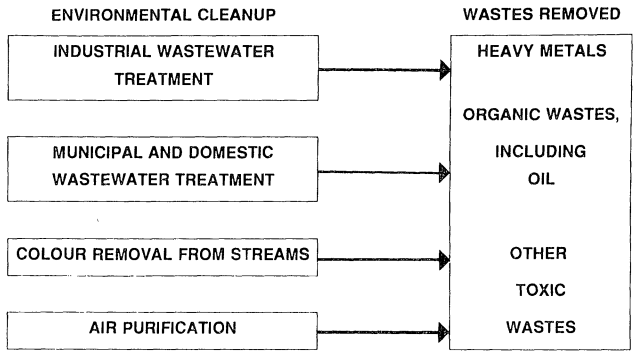


Figure 2. Potential of peat in the removal of pollutants.

2.2 MICROBIAL IMMOBILIZATION

The methods described by Mathiasson (1983) have been adapted for the immobilization of the microorganisms on the solid peat support. The microorganisms are continuously introduced and recycled through the filtration chamber during the required time to obtain viable cell attachment. Immobilization and biofilm formation are considered successful when a low concentration of free cell washout, as compared with the total biomass immobilized on the filter surface, is detected.

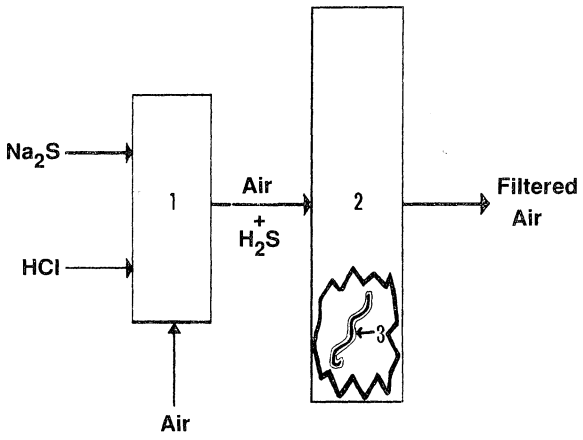


Figure 3. Diagram of a gas filtration system using peat. 1: H_2S generator. 2: Peat filter. 3: Biofilm.

2.3 ANALYTICAL METHODS

Gas chromatography is used to determine the efficiency of the biotransformation of the targeted pollutants by the biofilms, and to test for the presence of microbial metabolites, indicative of precursor compound biodegradation, some of which could also possess toxic characteristics. Michaelis Menten kinetics is employed to predict microbial degradation rates using a multiphasic mathematical model presented by Lewis *et al.* (1984). Microbial immobilization on the supporting peat fibres is determined by the direct counts method and by a modification of the adenosine 5' triphosphate (ATP) assay (Karl, 1980). Analysis of the composition of the peat

employed in the filters, before and after the biodegradative processes, is conducted by standard methods (Martin & Manu-Tawiah, 1989).

3. Results and Discussions

3.1 REMOVAL OF H_2S IN THE PEAT FILTERING UNIT

A diagram showing the removal of H_2S from a gaseous effluent by the peat-immobilized biofilm is shown in Figure 4. As Furusawa *et al.* (1984) observed, the peat *per se* has the ability of adsorbing gases, which is shown by the reduction of the concentration of H_2S in the effluent of a sterilized-peat filter. However, after the peat fibres have become saturated by the gas, the concentration in the effluent begins to rise until it reaches the same concentration as in the inlet. In the case of the non-sterilized peat fibres, the concentration of H_2S in the effluent decreases until a steady state is reached between the supply of the gas and the utilization of it by the biofilm on the peat fibres. This biodegradation process should be able to continue as long as the microorganisms can obtain other essential nutrients, such as N and P, from the environment.

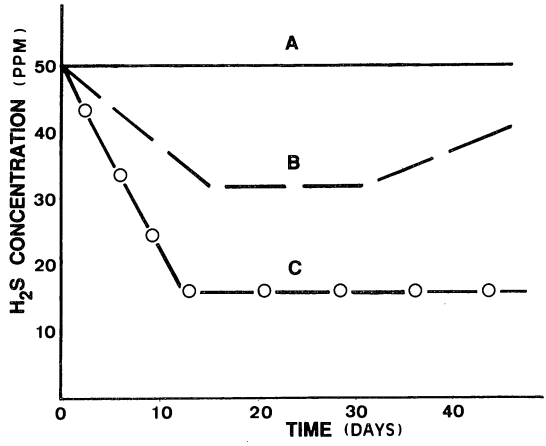


Figure 4. H_2S removal from air by a peat biofilter. A: Inflow H_2S concentration. B: Outflow H_2S concentration in a sterilized peat filter. C: Outflow H_2S concentration in a peat filter with biofilm.

3.2 PEAT AS A NUTRIENT SOURCE FOR MICROORGANISMS

Peat is an organic material composed of carbohydrates, minerals and a group of substances identified as humic acids, among other components. The complex mixture of organic compounds in peat and its relatively low price make this material a potential source of economical nutrients for microorganisms (Martin, 1984). Peat extracts have been used as the main media in the submerged culture of yeasts (Quierzy *et al.*, 1979) and fungi (Boa & LeDuy, 1982; Martin & White, 1986). The abovementioned information has indicated the properties of peat that allow its use as a support and substrate source for microorganisms present in a peat-based biofilm. In addition to acting as a support for the biofilm, it is expected that the peat should provide the microbial population with the additional nutrients required for its metabolic activities. Those nutrients could be either components of the peat that have been shown to be metabolizable by microorganisms, or nutrient supplements that have been also added to the peat. Figure 5 presents a schematic representation of this process. Table 1 shows the concentrations of nutrient materials in peat and peat extracts available for attached microorganisms (biofilms).

3.3 FURTHER STUDIES OF THE IMMOBILIZATION SYSTEM DESIGN

In addition to the microbial degradation process, the performance of the peat packed column system need to be studied, with the objective of improving its operational performance and creating the basis for the future optimization of the biodegradative system. The following aspects will be investigated: a) selection of the appropriate compaction of the column, considering the variables of substrate flow and residence time for effective degradation reaction; b) mass transfer of toxicants, nutrients (if added to the media) and potential metabolites, through the system, and; c) fouling of the immobilization media. Established techniques and mathematical models will be employed to assess these operating conditions. Light and electron microscopy will be used to assess the occurrence of fouling. When required, a cleaning process to remove fouling and contamination will be conducted by circulating sterile water and liquid peat extract solutions (Martin & Manu-Tawiah, 1989). The effective operating time of the peat columns will be also determined.

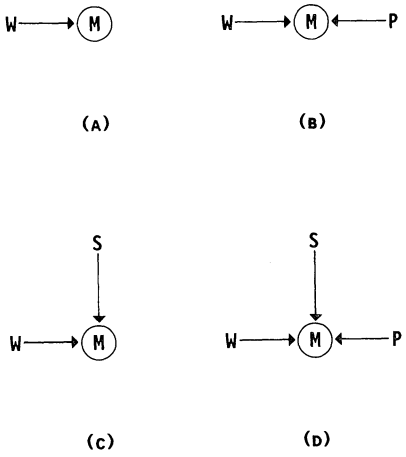


Figure 5. Different alternatives for the supply of nutrients to a degrading microbial population immobilized in a peat biofilter: (A) waste stream [W] as the only nutrient source; (B) waste stream and peat [P] as nutrient sources; (C) waste stream and substrate attached to the peat [S] as nutrient sources; (D) waste stream, peat and substrate attached to the peat as nutrient sources. Potentials for cometabolic reactions are present in B, C and D.

4. Acknowledgments

The work has been supported in part by a research grant from the Natural and Engineering Research Council of Canada.

Table 1: Mean concentration of nutrients in peat and peat extracts.

	Minerals	Carbohydrates	Lipids	Nitrogen
Peat[a]	4.5	89.7	5.1	0.7
Peat extracts[b]	3.0	34.3	2.0	0.7

[a] % dry weight
[b] Calculated by difference
[c] g/L

5. References

Boa, J.M. & LeDuy, A. (1982) 'Acidophilic fungus SCP from peat hydrolysate', Can. J. Chem. Eng., 60, 532-537.

Furusawa, N., Togashi, I., Hirai, M., Shoda, M. & Kubota, H. (1984) 'Removal of hydrogen sulphide by a biofilter with fibrous peat', J. Ferment. Technol., 62, 589-594.

Jaouich, B.A. (1975) 'Nitrate Reduction in Peat', Ph.D. thesis, University of Minnesota, Minneapolis, MN.

Karl, D.M. (1980) 'Cellular nucleotide measurements and applications in microbial ecology', Microbiol. Reviews, 44, 739-796.

Lewis, D.L., Holm, H.W. & Hudson, R.E. (1984) 'Application of single and multi-phasic Michaelis Menten kinetics to predictive modelling for aquatic ecosystems', Environ. Toxic. Chem. 3, 563-574.

Lochhead, A.G. & Thexton, R.H. (1947*a*) 'Growth and survival of bacteria in peat, I. Powdered peat and related products', Can. J. Res., 25, 1-13.

Lochhead, A.G. & Thexton, R.H. (1947*b*) 'Growth and survival of bacteria in peat. II. Peat pellets', Can. J. Res., 25, 14-19.

Mathiasson, B. (1983) 'Immobilization Methods', in B. Mathiasson (ed.), Immobilized Cells and Organelles, CRC Press, Inc. Boca Raton, Fla., pp. 3-25.

McLellan, J.K. & Rock, C.A. (1986) 'The application of peat in environmental pollution control: a review', Int. Peat J., 1, 1-14.

Martin, A.M. (1984) 'Study on the acid hydrolysis of peat: 1. Extraction of carbohydrates', J. Chem. Tech. Biotechnol., 34B, 70-74.

Martin, A.M. (1991) 'Peat as an agent in biological degradation: peat biofilters', in A.M. Martin (ed.), Biological Degradation of Wastes, Elsevier Science Publishers, London, pp. 341-362.

Martin, A.M. & Manu-Tawiah, W. (1989) 'Study of the acid hydrolysis of peat: composition of the extracts from *Sphagnum* peat', J. Chem. Technol. Biotechnol., 45, 171-179.

Martin, A.M. & White, M.D. (1986) 'Studies on the production of *Scytalidium acidophilum* biomass', Appl. Microbiol. Biotechnol., 24, 84-88.

Mueller, J.C. (1972). 'Peat in pollution abatement', in, Proceedings of the Symposium Peat Moss in Canada, University of Sherbrooke, Sherbrooke, Quebec, Canada, pp. 274-289.

Netzer, A. & Hughes, D.E. (1984) 'Adsorption of copper, lead and cobalt by activated carbon', Water Res., 18, 927-933.

Nichols, D.S. & Boelter, D.H. (1982) 'Treatment of secondary effluent with a peat-sand filter bed', J. Environ. Qual., 11, 86-92.

Pomeroy, R.D. (1982) 'Biological treatment of odourous air', J. Water Poll. Cont. Fed., 54, 1541-1545.

Quierzy, P.; Therien, N. & LeDuy, A. (1979) 'Production of *Candida utilis* protein from peat extracts', Biotechnol. Bioeng., 21, 1175-1190.

Smith, K.A.; Bremner, J.M. & Tabatabai, M.A. (1973) 'Sorption of gaseous atmospheric pollutants by soils', Soil Sci., 116, 313.

Wada, A.; Shoda, M.; Kubota, H.; Kobayashi, T.; Katayama-Fujimura, Y. & Kuraishi, H. (1986) 'Characteristics of H_2S oxidizing bacteria inhabiting a peat biofilter', J. Ferm. Technol., 64, 161-167.

Chapter 8

BIOFILMS IN WATER DISTRIBUTION AND INDUSTRIAL SYSTEMS

BIOFILMS IN DRINKING WATER DISTRIBUTION SYSTEMS

J.C. BLOCK
Environmental Health Laboratory
Faculté de Pharmacie
5 rue A. Lebrun
54000 Nancy, France

The degradation of water quality due to bacterial growth in the drinking water distribution systems is a major concern for consumers as well as for drinking water producers. Microorganisms (bacteria, yeasts, fungi, protozoa) may be found both in the water phase and on the surface of the pipe walls in the form of a biofilm.These thin layers (apart from deposits or tubercules) which are firmly anchored to the support and protect micro-organisms to a network of exopolymers composed of proteins and polysaccharides, form an ecosystem which is both stable and difficult to remove.
Conclusions of Van der Wende *et al.* (1989) or LeChevallier *et al.* (1987, 1988), as well as calculations based on the results published by Haudidier *et al.* (1988), show that in a potable water distribution system, even in the absence of chlorine, bacterial growth in the liquid phase is negligible. Essentially, only the bacteria in the biofilm attached to the walls of the distribution pipework are multiplying and, due to shear loss, constitute one of the main causes of the deterioration of microbiological quality of water distribution systems.
The occurrence of biofilms can cause a multitude of problems in drinking water distribution systems including :
- bacteria can be the starting point of a trophic food web leading to the proliferation of undesirable higher organisms ;
- specific bacterial species may generate turbidity, taste and odors in the drinking water ;
- high counts of heterotrophic bacteria (HPC) interfere with the detection of coliforms or sanitary indicators ;
- accumulation of attached biomass promotes biocorrosion ;
- biofilms increase frictional resistances thus reducing the capacity of distribution systems to carry water ;
- continued failure of the distribution system to meet all established water quality criteria (for coliforms or for heterotrophic plate counts).

Consequently, the control of biofilm accumulation requires several types of actions in combination (both at the level of the water treatment plant and of the distribution system itself) in order to achieve biologically stable drinking water distribution systems.
As shown on the figure 1 control of biofilm has usually been attempted by application of biocides in the water column. Because of the relative unefficiency of classical disinfectants (*e.g.* chlorine or chloramine), it is now recognized that strategies to control of attached biomass must involve several techniques (limiting biologically degradable organic carbon (BDOC) and the concentration of suspended bacterial cells in the water entering the distribution system) and then control methods must be based on data generated both from biofilm experiments and field studies.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 469–485.

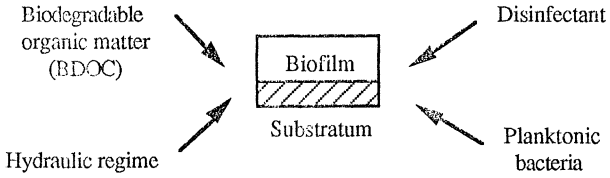


Figure 1. Some parameters governing biofilm accumulation in pipes.

Since 1985, active research has been carried out in Europe and the U.S.A. on this problem by research laboratories funded by the industry (in France : C.G.E., Lyonnaise des Eaux, S.A.U.R...), associations (A.W.W.A.,...), federal agencies (U.S.E.P.A., Agences de Bassin...), network of laboratories (European network "Biodegradable organic matter")... This paper will review some of the numerous publications and will analyse strategies for controlling biofilm bacteria from the perspective of the drinking water industry.

1. The real world !

Drinking water distribution networks are inherently huge and heterogeneous systems built to satisfy two rather conflicting requirements :

- on one hand to distribute water of a quality conforming to existing legal potable standards, although less than 4% of the water distribution will be used for human consumption in the strict sense ;
- on the other hand, to ensure that at every point within the supply area, sufficient quantity is available to meet the either constant demand or transient needs (fire-fighting, street cleansing, refilling of swimming pools).

The water residence time in the network is on average between 48h to 1 month based on the distance of the houses to the plant, the number of intermediary reservoirs, etc.
Irrespective of the quality of the distributed waters, this residence time is enough for chemical and biological reactions. Sampling of water distribution systems shows a drastic evolution in the water quality (Table 1).

TABLE 1. Variations of some parameters during distribution.
(↑ : increase ; ↓ : decrease)

Variations ↑	Variations ↓
Temperature	Chlorine
Conductivity	pH
Fluorescence	UV
Bacteria	DOC/BDOC

The methods reported by Maul *et al.* (1985) allows one to determine the spatial and temporal distribution of total heterotrophic bacteria in the network. A non-hierarchical nearest-centroid clustering method was used for dividing one water distribution system of the city of Metz, France, into zones corresponding to different levels of suspended bacterial den-

sity (Figure 2). The general pattern of the spatial heterogeneity showed a high degree of reproducibility. Since the frequency distribution of total heterotrophic bacteria within the zones was compatible with the negative binomial distribution, the water distribution system studied was considered as being composed of several heterogeneous subsystems. The consistency of this structured spatial dispersion pattern of bacteria in light of some physical and chemical characteristics of the system is evident. In consideration of the principal features of flow in the system relevant to the layout of water mains, the location of zones of highest bacterial concentrations have been attributed to lower levels of chlorine residuals and prolonged retention time of the water in the network, especially in the storage units. Although the monthly variation in the bacterial concentration of the entire system showed a marked increase which coincided with increasing water temperatures, the bacterial densities in each zones were subject to noticeable discrepancies in temporal variation.

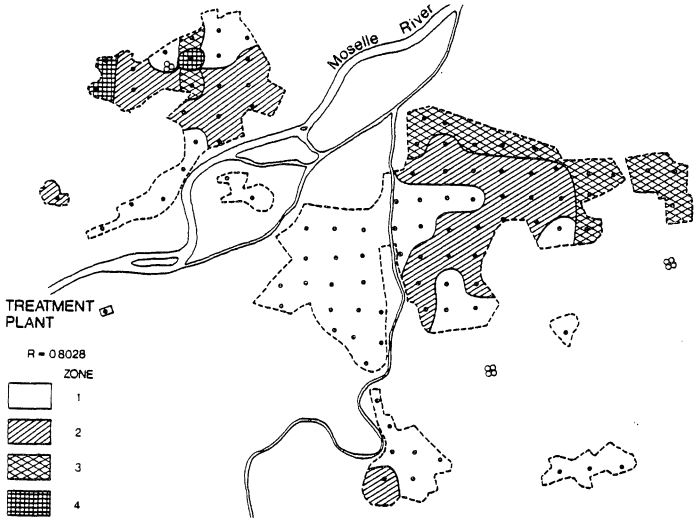


Figure 2. Spatial distribution of suspended bacterial counts in one water distribution system of the city of Metz, France.

In the same study, gram-negative bacteria isolated on R_2A agar medium (Reasoner and Geldreich, 1985) formed the dominant group in all zones. The relative cell number decreased distinctly from January to June 1984 (from 91.3% to 68.1%), and the number of gram-negative bacillae increased (from 7.8% to 29.2%). Those changes can be attributed to a decrease in relative *Pseudomonas* populations and an increase in *Corynebacterium* (whereas *Flavobacterium* appeared to remain quantitatively stable). Distribution of types of bacteria in the system was not homogeneous, and certain zones appeared to be dominated by particular species, (*i.e.*,*Corynebacterium*, etc). The public distribution system is thus an enormous heterogeneous reactor in which the different zones behave almost independently, especially regarding the diversity of bacterial populations.

Clearly then sampling design must be used in quality monitoring to determine whether or not the mean bacterial density of the water exceeds a specific standard. A set of criteria are required to determine the location and the optimal number of samples (Maul *et al.*, 1991). However, rational sampling procedures are still very rare and the pathway of the circulation of the water (flow rate, direction) is never perfectly known.

Such limited knowledge does not allow one to describe the complexity of actual water distribution network.

2. Microbiology of drinking waters

The microbiological analysis of water is commonly carried out by methods which have been in everyday use for decades and which, although not well-standardized, seem to be both simple and reliable in practice. However, it must be noted that the use of agar media for estimating bacteria in water samples invariably produces results which underestimate the numbers present and is related to the true value only by proportionality factors which are usually unknown and which depend on both the population of bacteria studied (its physiological state) and the culture methods used.
Within a bacterial population (either of a single species or a mixture of species) one finds variable numbers of bacteria which may be either living or dead, or in a less active state in between these two physiological extremes. Figure 3, which is adapted from the physiological classification discussed by Mason *et al.* (1988), shows a series of characteristic states which constitute intermediate stages between actively living bacteria and dead cells.

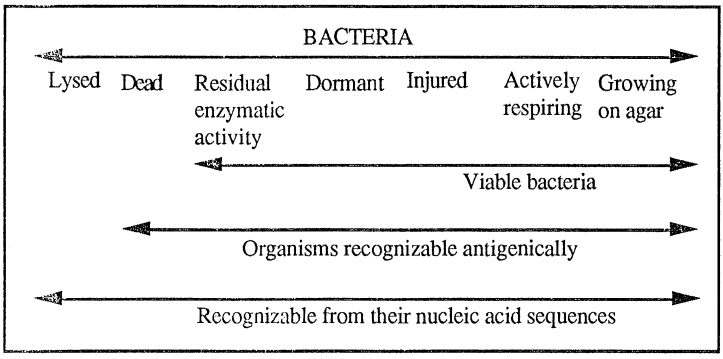


Figure 3. Classification of bacteria with respect to their physiological states, and methods of recognition.

The terms injured or dormant bacteria cover a rather poorly defined area, where either the nature of the injury or the degree of the dormancy are unknown. The only certain fact is that these injured or dormant bacteria are metabolically active but nevertheless incapable of replication on culture media. In general, as recently pointed out by Roszak and Colwell (1987), there is no method to distinguish with certainty between these different physiological states. In addition, available culture methods for waterborne bacteria give results which are well below the actual total cell count and which vary, however slightly, with the culture method modified. For example, figure 4 shows that there is a factor of 1000 separating the total number of active bacteria and the number of colony-forming units obtained by cultivation on agar media.
Like everything else around us, water normally contains living organisms (bacteria, fungi, macroinvertebrates, etc.). The drinking water treatment process, where it is necessary, is not intended to produce water which is sterile, but simply to render it pleasant to drink (clear and free of any unpleasant taste and odor) and non-injurious to health. For this rea-

son, it is possible to find many populations of living organisms within the water contained in the network. A subjective classification into four groups can be proposed :

- indigenous species : these are composed mostly of microorganisms which are also found in the soil, on food, etc., such as bacteria, yeasts and microscopic fungi ;

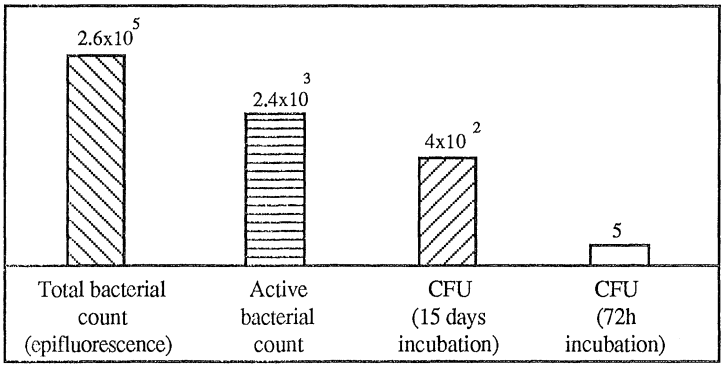


Figure 4. Numbers of bacteria in disinfected drinking water supplies at the outlet from the treatment plant.

- species which arrive unexpectedly at the consumer's tap. They may include organisms such as protozoa and macroinvertebrates of the genus *Asella* which, when accidentally introduced into the mains network, can survive and multiply to a level of several thousands per cubic metre (Boet, 1984 ; Levy *et al.*, 1986) ;
- nuisance species, which aggravate the problem of chemical corrosion or give rise to an unpleasant taste in the water. In the latter case, the presence of bacteria of the genus *Actinomycetes*, capable in some circumstances of secreting such molecules as geosmin, can impart a very disagreable taste to the water, even at low concentrations. These species are also a nuisance if the extent of their multiplication is such as to mask the detection of a potentially dangerous species ;
- dangerous species can be injurious to health if they consist of human pathogens which have entered the network as a result of accidental contamination.

All the aquatic microorganisms habitually encountered in river water can be isolated from potable water provided the sample is first concentrated, due to the small number of organisms present.

Water distribution networks constitute an ecosystem in which the selection pressures comprise, in particular, relatively low concentration of nutrients and the presence, from time to time, of toxic substances such as chlorine and its derivatives, metal ions, etc. It is no surprise that the literature lists numerous of bacterial species and genera in water systems, of which Table 2 provides a simple and non-exhaustive illustration. Clearly one can say that the majority of bacteria typical of freshwater ecosystems can be found among the flora of water distribution systems, although such organisms are present at very low concentrations per litre or per cubic metre. In fact, it is very rare for surveys to attempt to describe the entire microflora. Actually, most studies have a well-defined objective ; to demonstrate the occurrence of a specific genus or species of bacteria. Despite the widely differing results, it is possible to draw some conclusions of a general nature.

(1) In the case of waters which have been correctly treated, the frequency of isolation of indicator organisms for faecal contamination (*Escherichia coli*, *Streptococcus faecalis*) is very low. By way of example, less than 0.3% to 2% of samples obtained from the water supply network in the Paris region exhibited detectable levels of such indicator organisms (Bourbigot *et al.*, 1984). However, non-faecal coliforms (*Enterobacter*, *Citrobacter*, *Klebsiella*) were detected more often (Geldreich, 1986 ; Geldreich and Rice, 1987).
This situation is perfectly in order with public health objectives and is typical of urban environments and localities which are fairly densely populated and stringently monitored. However, for sparsely populated localities, instances of non-compliance with public health regulations are readily observed (even in the most highly industrialized countries) and quite common (Collin *et al.*, 1981 ; Sworobuk *et al.*, 1987).

TABLE 2. Some typical microorganisms found in drinking water.

Potential pathogens and bacterial indicators	Autochtonous bacteria	Corrosion bacteria	Yeasts and fungi
Salmonella,...	*Acinetobacter*	Sulfato-reductor	*Penicillium*
	Aeromonas	bacteria	*Rhizopus*
	Alcaligenes		*Mycelium*
	Bacillus		*Trichomonas*
Enterovirus	*Enterobacter*	Iron bacteria	*Mucor*
	Flavobacterium		*Aspergillus*
	Micrococcus		
	Pseudomonas		
E. coli, *Streptococcus*	*Staphylococcus*		
	Corynebacterium		
	Photobacterium		
	Proteus		
Legionella	*Yersinia*		

(2) A harmless flora is always conveyed in delivered water, varying in amounts (depending on the site of origin) from 10^2 to 10^5 bacterial cells (living and dead) per millilitre and from 1 to 10^3 colony-forming units per millilitre (Dollard *et al.*, 1985 ; Fransolet *et al.*, 1986 ; Korsholm and Søgård, 1987 ; Maki *et al.*, 1986 ; Schœnen, 1986a). This flora, which may be quantified either by direct counts using either epifluorescence techniques (Delattre, 1986) or by culturing on a nutrient agar (total heterotrophic plate count) is very often dominated by gram-negative bacteria (O'Connor and Banerji, 1984 ; Olson and Nagy, 1984). Among the most frequently occurring forms are bacteria of the genus *Pseudomonas* and the yellowish-brown pigmented *Flavobacterium*-like rod-shaped bacteria (Dollard *et al.*, 1985 ; Fransolet *et al.*, 1986 ; LeChevallier *et al.*, 1987a ; Maki *et al.*, 1986 ; Oger *et al.*, 1987 ; Scarpino *et al.*, 1987 ; Van der Kooij, 1977).

(3) Along with this regularly occurring harmless flora in the water supply network, pathogenic or potentially pathogenic organisms may be sporadically detected, such as *Legionella* (Habicht and Müller, 1988 ; Hsu *et al.*, 1984 ; Shands *et al.*, 1985 ; Voss *et al.*, 1985), *Staphylococcus aureus* (Lamka *et al.*, 1980 ; LeChevallier and Seidler, 1980), *Yersinia enterocolitica* (Schindler, 1984). Such occurrences do not always coincide with epidemics of, for example, gastroenteritis, sickness, etc., in the population served, and conversely a number of epidemic incidents, such as common gastric

upsets, affecting the consumer population can never be traced back to any specific aetiological agent (Collin *et al.*, 1981).

(4) While bacteria may be the microorganisms present in greatest abundance in the distribution network, they are by no means the only inhabitants. Yeasts and microscopic fungi may be present in concentrations ranging from 1 to 10^4 per litre (Burman and Colbourne, 1977 ; Hinzelin and Block, 1985 ; Nagy and Olson, 1986 ; Rizet *et al.*, 1985 ; Rose *et al.*, 1988 ; Rosenzweig and Pipes, 1988). The algal cells (diatoms, *Chlorophyceae*) carried through the system from the source can reach concentrations as high as 10^4 to 10^6 per litre (Rizet *et al.*, 1985). Protozoa of the genus *Amœba* and flagellates are also normally present in all distribution networks.

3. Biofilm of distribution netwoks

Viewed as a reactor, a potable water distribution network resembles a two-phase system of which one phase is the water and the second phase consists of the solid pipework wall and the solid suspended cell particles entrained in the flowing water (Figure 5).

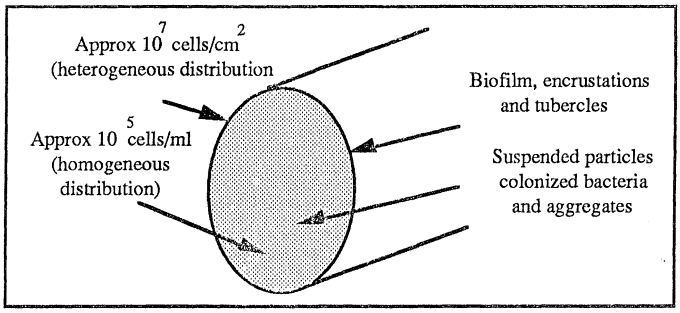


Figure 5. Schematic diagram of a water main pipe and the distribution of microorganisms.

At least 50% of the bacteria carried by the water are present as aggregates of dimensions greater than 5 µm or attached to non-biological particles of over 5 µm diameter, *e.g.* grains of sand or active carbon (McFeters *et al.*, 1987 ; Ridgway and Olson, 1981). These particles, as soon as they come into contact with water, become colonized by microorganisms from the aquatic environment. This biomass (bacteria, microscopic fungi, protozoa, yeasts, etc.) is attached to either the walls of the pipework in a heterogeneous pattern determined by the shear-forces acting on the wall to the precipitates, deposits of sediment, encrustations and tubercles which form on the pipe wall surface (Allen *et al.*, 1980 ; LeChevallier *et al.*, 1987a ; Ridgway and Olson, 1981).

Hence the generic term "biofilm", which often describes the presence of microorganisms on the surface of submerged materials, should not imply in any sense the existence of a homogeneous film of regular thickness. The biofilm should simply be regarded as an accumulation, more or less evenly spread over the wall of the immersed support, of living and dead microorganisms and polymers and/or macromolecules excreted by the biomass.

Thus, fixed-film bacterial population densities can range from 10 to 10^8 CFU/cm^2 (Donlan and Pipes, 1986 ; Haudidier *et al.*, 1988 ; LeChevallier *et al.*, 1987) and for microscopic fungi from 46 to 250 UFC/cm^2 (Nagy and Olson, 1986). Olson (1983) endeavoured to

obtain an exponential relation between the quantity of attached organisms and the history of the site but was unable to distinguish between the effect of age and that of the physico-chemical nature of the pipe.
In an experimental network, Block *et al.* (1992a) found that the attached biomass was 10 times higher than the planktonic biomass. Biofilms were composed of a complex mixture of microorganisms : fungi, yeasts, protozoa (*Bodo* sp), free amœba (*Hartmannella*, *Vannella*, *Cochliopodium* and *Nægleria*), bacterial cells (up to 10^6 cells/cm^2), 14% of which were actively INT respiring bacteria (Figure 6).

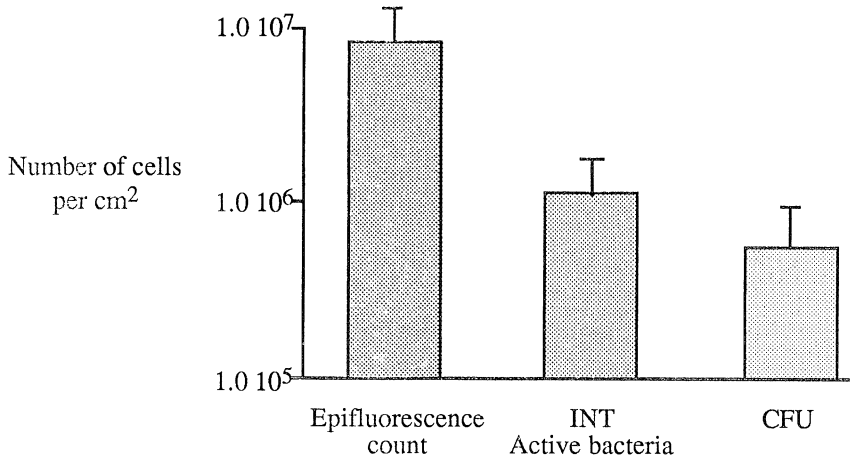


Figure 6. Number of total, active and heterotrophic bacteria attached to cast iron pipe lined with cement (Block *et al.*, 1992a).

4. Parameters governing the accumulation of biofilms

The formation of a bacterial biofilm is governed by at least four factors which are already well known (Bryers, 1987 ; Bryers and Characklis, 1982 ; Characklis, 1990 ; Lawrence and Caldwell, 1987 ; Trulear and Characklis, 1982). These are :
- deposition and adsorption of both living and dead microorganisms from the aqueous phase onto the solid phase ;
- growth, in the true sense of the term, of attached microorganisms in the active state at the expense of biodegradable organic matter in the water (with formation of new cells and, secreted by the cells, exopolymers which help to ensure the cells'firm attachment) ;
- the death of attached microorganisms ;
- continual erosion of the biomass by the flow of the water.

Strictly the term "biofilm net accumulation" should be employed rather than that of "growth of biofilm", which in the actuality refers to only one of the several fundamental processes contributing to the overall phenomenon. Clearly the biofilm present in a water network is a dynamic system which is constantly being broken down and re-established. As already discussed, the growth of bacteria within the network, when it takes place, occurs almost entirely in the biofilm and not in the water phase.
The characteristics of the biofilm (species present, number of cells, specific gravity and thickness) are thus controlled by a myriad of factors including the number and diversity of

the species present in the water, the concentration and nature of the biodegradable organic matter in the water, the hydraulic regime to which the system is subjected, and the characteristics of the support material colonized by the bacteria.

4.1. DEPOSITION ADHESION OF MICROORGANISMS

The water distribution network is permanently contaminated by a suspended mixed flora which will partly adsorb onto the pipe walls. Adhesion of bacterial cells occurs on all kinds of substratum in contact with water. After several days of immersion it is possible to rank different materials according to the number of attached cells : cast iron > tinned iron > cement lined cast iron > stainless steel (Block *et al.*, 1991). However the relationship between the different surface characteristic descriptors and the number of attached bacteria depends on the descriptor considered : wettable area, roughness, tumultuosity.
E. coli experimentally introduced from one single rapid injection into a drinking water distribution system pilot plant adsorbed around 50% to the indigenous biofilm in 1 hour (Fass and Block, 1992) (Figures 7 and 8).

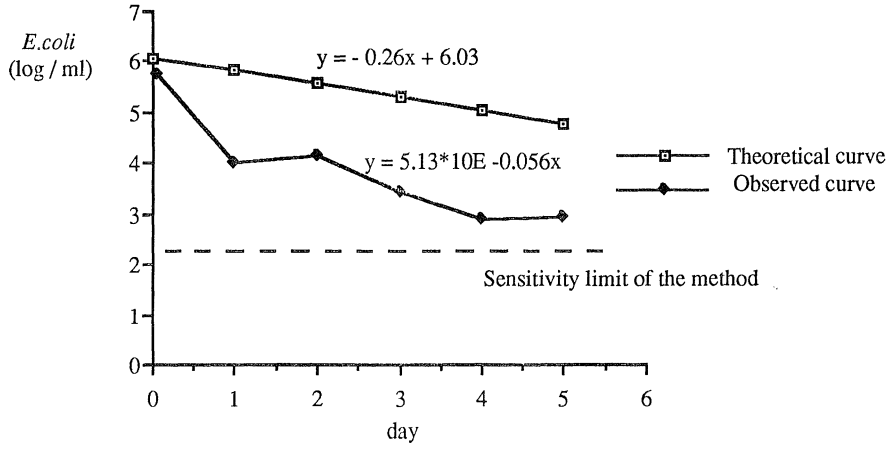


Figure 7. Pattern of injected *E. coli* in the water of an experimental water recycle loop (Fass and Block, 1992).
_____ theoretical curve of injected cell washout assuming no adsorption
_____ observed curve (daily data from 1 hour after injection to the 5th day).

Attached bacteria may then behave in two ways :
(i) bacteria are not able to multiply in the network and are thus washout from the distribution system (water + biofilm) will be inevitable and controlled by the flow rate entering the system and by the resulting dilution rate ;
(ii) bacteria are able to mutiply in the network and will maintain to a certain concentration depending their affinity for nutrients, the availability of organic matter.

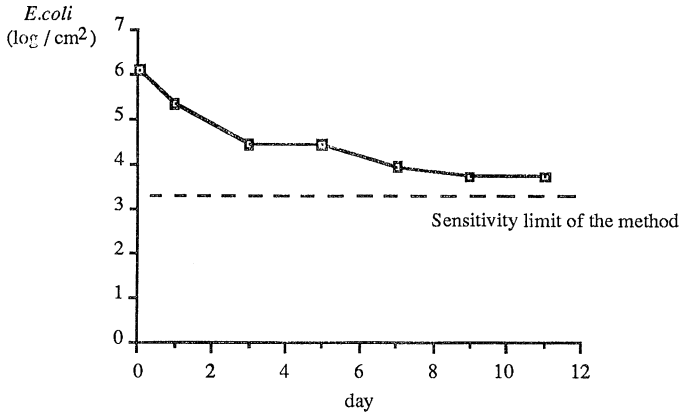


Figure 8. Pattern of injected *E. coli* accumulated in the biofilm (data are presented from the point "1 hour after injection" to day 11) (Fass and Block, 1992).

By way of consequence one may recognize that the limitation of the flux of cells entering the network will also decrease the density of the biofilm. The figure 9 shows that eliminating suspended cells entering the network (planktonic) will decrease the biofilm population by 100 or 1000 folds !

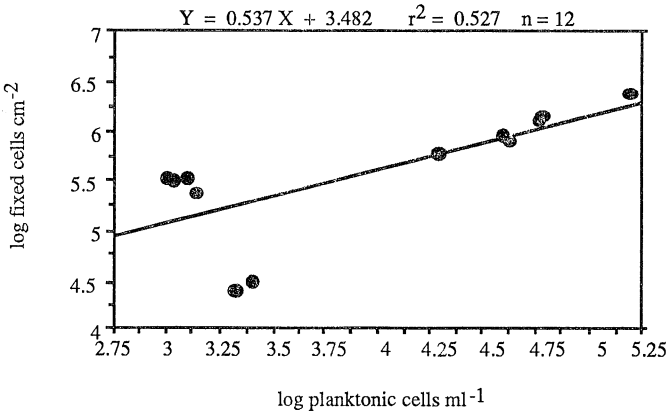


Figure 9. Relationship between the cell density in finished water (cells ml^{-1}) entering the network and the density of fixed cells (cells cm^{-2}) in loops 1 (chlorinated and chloraminated networks) (cells cm^{-2}) (Mathieu, 1992).

4.2. GROWTH OF ATTACHED BACTERIA

Growth of bacteria requires biodegradable nutrients generally measured as the biodegradable dissolved organic carbon - BDOC (Block *et al.*, 1992b). Such biodegradable compounds may be carried by the water flow or leached from distribution system materials which must be avoided (Rittmann and Snœyink, 1984).
Finished waters contain from 1 to 5 mg/l of DOC, 30% of which are biodegradable.
Several models of the dynamics of BDOC and bacteria in distribution networks have been recently presented. A number of existing models can be solved to predict both the impact

of BDOC on the biological stability of the waters and the number of bacteria (HPC or coliforms in the network) including :

. LeChevallier *et al.* (1991)	Number of cells + chlorine + temperature
. Desjardins *et al.* (1991)	Flow rate + volume + area of the pipes initial + DOC + kinetic constant for DOC removal
. Servais *et al.* (1991)	Bacterial adsorption + desorption processes + bacterial attachment + chlorine
. Mathieu (1992)	Number of cells + chlorine + DOC
. Joret and Volk (pers com, 1992)	DOC + time + number of bacteria

From Haudidier *et al.* (1988), we learn that BDOC is rapidly removed in the absence of chlorine and this is especially true in pipes with a small diameter (I.D. ≤ 10 cm) (Servais *et al.*, 1991). The direct consequence of the biodegradation/assimilation of organic matter is an increase of the number of cells in the biofilm and subsequently in the water phase.
Characterization of the biofilm by its specific growth rate can be obtained from appropriate experiments leading to the calculation of each process rate contributing to biofilm formation (Bryers and Characklis, 1982) or by applying the simplified model previously used by Van der Wende *et al.* (1989). This model expresses the biofilm specific growth rate μ (h^{-1}) as

$$\mu = D\ (X_i - X_{i-1})\ V / X_b\ A \qquad (1)$$

in which X_i and X_{i-1} are the outlet and inlet suspended biomass concentrations (cells. ml^{-1}) ; D is the dilution rate (h^{-1}) ; X_b is the biofilm cell density (cells.cm^{-2}) ; V is the volume of the pipe (liters) ; A is the wetted surface of the pipe (cm^2). Such a model assumes that both the endogenous decay rate (km) and the planktonic growth rate are negligible. As demonstrated from previous experiments by Haudidier *et al.* (1988), in the absence of chlorine residual, km is relatively low (km = 0.0018 h^{-1}) and calculated apparent planktonic growth rates are also very low.
Substituting in equation 1, the values of X_b (as epifluorescence counts per area material coupons) concentrations of suspended cells (X_i and X_{i-1}), and the specific growth rate of the biofilm cells can be calculated for each pipe. As shown by Block *et al.* (1992a), the specific growth rate of cells attached to cement is high (μ = 0.0017 h^{-1}) in the first section of a pilot scale water supply network which received the largest flux of organic matter. This translates to a cell generation or doubling time in the biofilm (25°C, no chlorine) of approximatively 17 days.
The values of μ decrease drastically down the pilot-scale network and reached an average value of 0.0004 h^{-1} and 0.0001 h^{-1} (generation time equal to 72 days and 288 days respectively).
Changes of μ values can be related to high total organic carbon (TOC) consumption, especially at the beginning of the network (around 82% of the biodegradable organic matter is consumed after 40h). Calculated specific growth rates for PVC material are generally higher or equivalent to cement cast iron.

A threshold value of BDOC for biofilm growth ?
Theoretically one could propose a minimal value for the nutrients (Smin) to establish biofilm development, however practically concentrations as low as 100 µg per liter (Mathieu *et al.*, 1992) or 40 µg per liter (Mittelman, 1991) can sustain cell production (see Figure 10).

Which technic to use for BDOC removing ?
In spite of biological treatments which give better waters in terms of biological stability, some others technics (nanofiltration or...) will have to be developed in the next future in order to come to the zero BDOC !

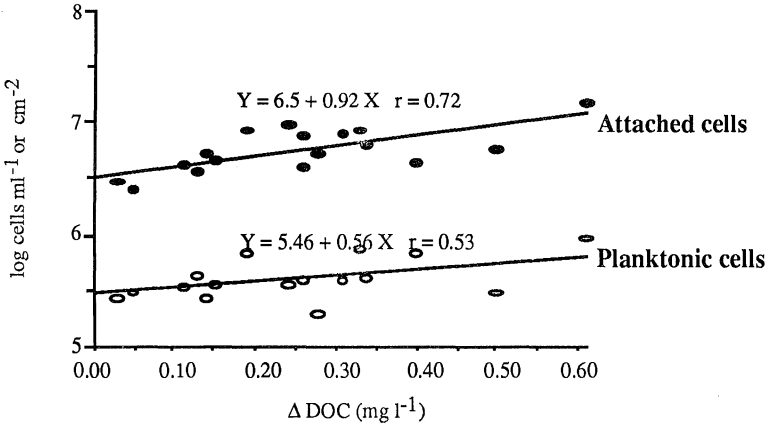


Figure 10. Relationship between the number of cells in network without post-disinfectant and the concentration of bioremoved DOC (Mathieu *et al.*, 1992).

4.3. BIOCIDE APPLICATION

Chlorine and chloramine while frequently used as post-disinfectants should not be a panacea. Both chemicals lead to the formation of disinfectant by-products and their efficiency against attached cells is limited (Figure 11).

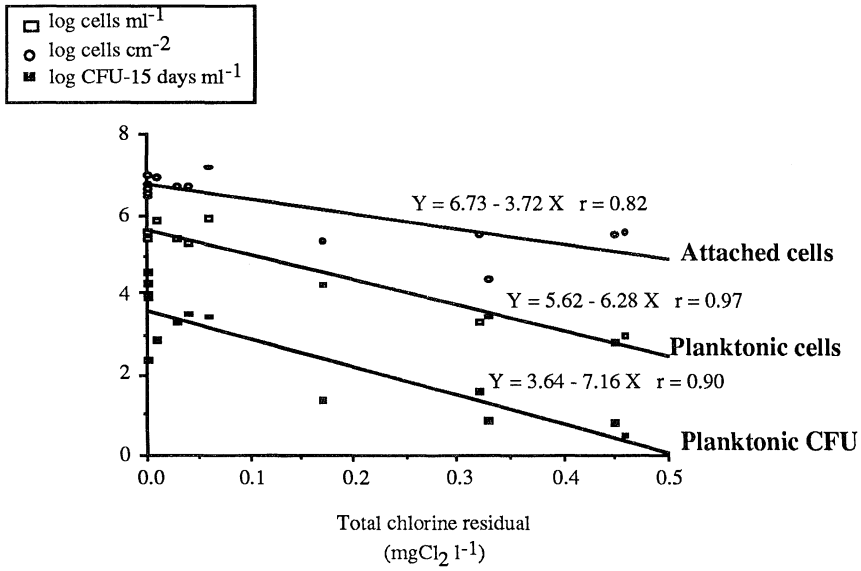


Figure 11. Relationship between the cell density and the residual chlorine in a post-chlorinated network (Mathieu *et al.*, 1992).

By extrapolation of experimental data, one may show (Table 3) that (i) it is quiet impossible to prevent biofilm accumulation in agreement with Characklis (1990) and Paquin *et al.* (1992) and (ii) chloramine is definitively less efficient than chlorine in spite of data presented by LeChevallier *et al.* (1990).

TABLE 3. Theoretical effective chlorine and chloramine concentration for post-disinfection (Mathieu *et al.*, 1992).

Objective	Residual chlorine ($mgCl_2/l$)	Residual chloramine ($mgCl_2/l$)
No biofilm	1.8	8.8
Cell-free water	0.88	4
Disinfected	0.51	1.3

5. Conclusions

In conclusion, one has to keep in mind that biofilm control in distribution system is complicated and requires continuous action. Above all the characteristics of the finished waters feeding the system have to be carefully controled (low BDOC, low cell concentration). A post-disinfection with chlorine is not a curative treatment but a additional precaution ; its efficiency is directly related to the previous organic matter reduction and a good hydraulic regime. As shown on Table 4 the challenge is still there !

TABLE 4. Some technics for achieving biologically stable waters.

Technic	Microfiltration	Biological treatment	Chlorination
Principle	Cell removal from finished waters entering the networks	Biological filters with attached biomass	Effective concentration of residual biocide
Objective	no cells	< 100 µg BDOC.l-1	> 0.5 mg Cl2.l-1

However, it would appear to be possible to minimize the use of chlorine as disinfectant by controlling of the organic matter which appears as a priority pollutant-like.

6. Acknowledgments

The author would like to thank all the researchers who have participated or are participating to this research project : S. Fass, L. Mathieu, A. Maul, J.L. Paquin... Our grateful thanks also go to the companies that have financed some of the works described here : Mosellane des Eaux, Compagnie Générale des Eaux, Pont-à-Mousson S.A., S.E.D.I.F., U.S.E.P.A... Rewritting of the paper by J. Bryers was greatly appreciated.

7. References

Allen, M.J., Taylor, R.H. and Geldreich, F.E. (1980) 'The occurrence of microorganisms in water main encrustations', J. Am. Wat. Wks Assoc., 72, 614-625.

Block, J.C., Haudidier, K., Paquin, J.L., Miazga, J. and Lévi, Y. (1992a) 'Biofilm accumulation in drinking water distribution systems', Biofouling (in press).

Block, J.C., Mathieu, L., Servais, P., Fontvieille, D. and Werner, P. (1992b) 'Indigenous bacterial inocula for measuring the biodegradable dissolved organic carbon (BDOC) in waters', Wat. Res., 26, 481-486.

Block, J.C., Paquin, J.L., Colin, F., Miazga, J. and Lévi, Y. (1991) 'Accumulation de bactéries sur différents types de matériaux : étude comparative en réseau de distribution', Séminaire Adhésion des Microorganismes aux surfaces solides, INA, Massy.

Boet, P. (1984) 'Mises au point sur la biologie du crustacé isopode *Asellus aquaticus* et sur les moyens de son éradication dans les réseaux de distribution d'eau potable', Sciences de l'Eau, 3, 93-110.

Bourbigot, M.M., Dodin, A. and L'Heritier, R. (1984) 'La flore bactérienne dans un réseau de distribution', Wat. Res., 18, 589-591.

Bryers, J.D. (1987) 'Biologically active surfaces : processes governing the formation and persistence of biofilms', Biotechnol. Progress, 3, 57-68.

Bryers, J.D. and Characklis, W.G. (1982) 'Processes governing primary biofilm formation', Biotechnol. Bioeng., 24, 2451-2476.

Burman, N.P. and Colebourne, J.S. (1977) 'Techniques for the assessment of growth of microorganisms on plumbing materials used in contact with potable water supplies', J. Appl. Bacteriol., 43, 137-144.

Characklis, W.G. (1990) 'Microbial biofouling control', in Biofilms, Characklis, W.G. and Marshall, K.C. (eds.), John Wiley and sons, New York, 589-633.

Collin, J.F., Melet, J.J., Morlot, M. and Foliguet, J.M. (1981) 'Eau d'adduction publique et gastro-entérites en Meurthe-et-Moselle', J. Fr. Hydrol., 12, 155-174.

Delattre J.M., (1986) 'Le contrôle bactérien rapide des eaux par épifluorescence', J. Fr. Hydrol., 17, 59-69.

Dollard, M.A., Schwartzbrod, J., Maul, A. and Block, J.C. (1985) 'Diversité des populations bactériennes d'un réseau de distribution publique', J. Fr. Hydrol., 16, 287-299.

Donlan, R.M. and Pipes, W.O. (1986) 'Pipewall biofilm in drinking water mains', 14th Annual AWWA Water Quality Technology Conference, Portland, Oregon.

Fass, S. and Block, J.C. (1992) 'Fate of *Escherichia coli* experimentally injected in a drinking water distribution system', Wat. Res. (submitted).

Fransolet, G., Villers, G., Goyens, A. and Masschelein, W.J. (1986) 'Metabolic identification of germs isolated from ozonized water mixed with underground water', Ozone Sci. Engng., 8, 95- 106.

Geldreich, E.E. (1986) 'Coliform non-compliance nightmares in water supply distribution systems', in Advances in water analysis and treatment, Proceeding of the AWWA Water Quality and Technology Conference, Portland.

Habicht, W. and Müller, H.E. (1988) 'Occurrence and parameters of frequency of *Legionella* in warm water systems of hospitals and hotels in lower Saxony', Zbl. Bakt. Hyg., B186, 79-88.

Haudidier, K., Paquin, J.L., Français, T., *et al.* (1988) 'Biofilm growth in a drinking water network : a preliminary industrial pilot plant experiment', Wat. Sci. Tech., 20, 109-115.

Hinzelin, F. and Block, J.C. (1985) 'Yeasts and filamentous fungi in drinking water', Environ. Technol. Letters, 6, 101-106.

Hsu, S.C., Martin, R. and Wentworth, B.B. (1984) 'Isolation of *Legionella* species from drinking water', Appl. Environ. Microbiol., 48, 830-832.

Korsholm, F. and Søgård, H. (1987) 'Colony counts in drinking water bacteriology - importance of media and methods', Zbl. Bakt. Hyg., B185, 112-120.

Lamka, K.G., LeChevallier, M.W. and Seidler, R.J. (1980) 'Bacterial contamination of drinking water supplies in a modern rural neighbourhood', Appl. Environ. Microbiol., 39, 734-738.

Lawrence, J.R. and Caldwell, D.E. (1987) 'Behavior of bacterial stream population within the hydrodynamic boundary layers of surface micro-environments', Microb. Ecol., 14, 15-27.

LeChevallier, M.W. and McFeters, G.A. (1985) 'Enumerating injured coliforms in drinking water', J. Am. Wat. Wks Ass., 77, 81-88.

LeChevallier, M.W., Babcock, T.M. and Lee, R.G. (1987a) 'Examination and characterization of distribution system biofilms', Appl. Environ. Microbiol., 53, 2714-2724.

LeChevallier, M.W., Schiemann, D.A. and McFeters, G.A. (1987b) 'Factors contributing to the reduced invasiveness of chlorine-injured *Yersinia enterocolitica*', Appl. Environ. Microbiol., 53, 1358-1364.

LeChevallier, M.W., Cawthon, C.D. and Lee, R.G. (1988) 'Factors promoting survival of bacteria in chlorinated water supplies', Appl. Environ. Microbiol., 54, 649-654.

Levy, R.V., Hart, F.L. and Cheetham, R.D. (1986) 'Occurrence and public health significance of invertebrates in drinking water systems', J. Am. Wat. Wks Ass., 78, 105-110.

Maki, J.S., La Croix, S.J., Hopkins, B.S. and Staley, J.T. (1986) 'Recovery and diversity of heterotrophic bacteria from chlorinated drinking waters', Appl. Environ. Microbiol., 51, 1047-1055.

Mason, C.A., Hamer, G. and Bryers, J.D. (1988) 'The death and lysis of microorganisms in environmental processes', FEMS Microbiol. Rev., 39, 373-401.

Mathieu, L., Paquin, J.L., Block, J.C., Hartemann, P. and Colin, F. (1992) 'Paramètres gouvernant la prolifération bactérienne dans les réseaux de distribution', Sciences de l'Eau (in press).

Mathieu, L. (1992) 'Matière organique biodégradable et stabilité biologique des eaux au cours de leur distribution', Ph D Thesis Université de Nancy I, Mention Chimie-Microbiologie de l'Eau, 219 pages.

Maul, A., El-Shaarawi, A.H. and Block, J.C. (1985) 'Heterotrophic bacteria in water distribution systems. I Spatial and temporal variation. II Sampling design for monitoring', Sci. Tot. Environ., 44, 201-224.

Maul, A., Vagost, D. and Block, J.C. (1991) 'Microbiological analysis in water distribution networks : sampling strategies, methods and computer programs', Ellis Horwood Publishers, New York, 127 pages.

McFeters, G.A., Camper, A.K., LeChevallier, M.W., Broadway, S. and Davies, D.G. (1987) 'Bacteria attached to granular activated carbon in drinking water', Environmental Res. Brief, June, 1-5, USEPA Cincinatti.

Mittelman, M.W. (1991) 'Bacterial growth and biofouling control in purified water systems', in Biofouling and biocorrosion in industrial water systems, Flemming, H.C. and Geesey, G.G. (eds.), Springer-Verlag, NY, 113-132.

Nagy, L.A. and Olson, B.H. (1986) 'A comparison of media for the enumeration of filamentous fungi from aqueduct biofilm', Zbl. Bakt. Hyg., B182, 478-484.

O'Connor, J.T. and Banerji, S.K. (1984) 'Biologically mediated corrosion and water quality deterioration in distribution systems', USEPA Report 600/2/84/056, Cincinnati, OH, 442 p.

Oger, V., Hernandez, J.F., Delattre, J.M., Delabroise, A.H. and Krupskay, S. (1987) 'Etude par épifluorescence de l'évolution de la microflore totale dans une eau minérale embouteillée', Wat. Res., 21, 469-474.

Olson, B.H. (1983) 'Microbiology of drinking water pipes', Communication 3rd International Symposium on Microbial. Ecology, East Lansing, Mich.

Olson, B.H. and Nagy, L.A. (1984) 'Microbiology of potable water', in Laskin, A.I. (ed.), Academic Press, Inc., London, Advances in Applied Microbiology, 30, 73-132.

Paquin, J.L., Block, J.C., Haudidier, K., Hartemann, P., Colin, F. Miazga, J. and Lévi, Y. (1992) 'Chloration et colonisation bactérienne d'un réseau de distribution expérimental', Sciences de l'Eau (in press).

Reasoner, D.J. and Geldreich, E.E. (1985) 'A new medium for the enumeration and subculture of bacteria from potable water', Appl. Environ. Microbiol., 49, 1-7.

Ridgway, H.F. and Olson, B.H. (1981) 'Scanning electron microscope evidence for bacterial colonization of a drinking-water distribution system', Appl. Environ. Microbiol., 41, 274-287.

Rittmann, B.E. and Snœyink, V.L. (1984) 'Achieving biologically stable drinking water', J. Am. Wat. Wks Ass., 76, 106-114.

Rizet, M., Depardieux, J., Gognet, M. and Lorentz, F. (1985) 'Populations microscopiques des réseaux de distribution d'eau potable : effet d'un nettoyage mécanique', J. Fr. Hydrol., 16, 269-286.

Rose, J.B., Gerba, C.P., Hayes, E., Matte, T. and Juranek, D.D. (1988) 'Occurrence of *Cryptosporidium oocysts* in a distribution system after a waterborne outbreak', IAWPRC Brighton Conference.

Rosenzweig, W.D. and Pipes, W.O. (1988) 'Fungi from potable water : interaction with chlorine and engineering effects' Proceedings of the International Conference on Water and Wastewater Microbiology, vol. 1, 257/258, Irvine, Ca.

Roszak, D.B. and Colwell, R.R. (1987) 'Survival strategies of bacteria in the natural environment', Microbiol. Rev., 51, 365-379.

Scarpino, P.V., Kellner, G.R. and Cook, H.C. (1987) 'The bacterial quality of bottled water', J. Environ. Sci. Health, A22, 357-367.

Schindler, P.R.G. (1984) 'Nachweis von *Yersinia enterolitica* aus Trinkwasserversorgungsanlagen in Südbayern', Zbl. Bakt. Hyg., I. Abt. Orig., B180, 76-84.

Schœnen, D. (1986a) 'Neuere Untersuchungen zur Wiederverkeimung des Trinkwassers', Zbl. Bakt. Hyg., B183, 70-78.

Schœnen, D. (1986b) 'Microbial growth due to materials used in drinking water systems', in Biotechnology, Rehm, H.J. and Reed, G. (eds.), VCH Publishers, Weinheim, chap. 20, 627-647.

Servais, P., Billen, G., Laurent, P., Lévi, Y. and Randon, G. (1991) 'Studies of BDOC and bacterial dynamics in the drinking water distribution system of the northern parisian suburbs', Séminaire International "Matière organique et réseaux de distribution", Nancy.

Shands, K.N., Ho, J.L., Meyer, R.D., Gorman, G.W., Edelstein, P.H., Mallison, G.F., Finegold, S.M. and Fraser, D.W. (1985) 'Potable water as a source of Legionnaires'disease', J. Am. Medic. Ass., 253, 1412-1416.

Sworobuk, J.E., Law, C.B. and Bissonnette, G.K. (1987) 'Assessment of the bacteriological quality of rural groundwater supplies in northern West Virginia', Water, Air, and Soil Pollution, 36, 136-170.

Trulear, M.G. and Characklis, W.G. (1982) 'Dynamics of biofilm processes', J. Wat. Poll. Control Fed., 54, 1288-1301.
Van der Kooij, D. (1977) 'The occurrence of *Pseudomonas* spp. in surface water and in tap water as determined on citrate media', Antonie van Leeuwenhœk, 43, 187-197.
Van der Wende, E., Characklis, W.G. and Smith, D.B. (1989) 'Biofilms and bacterial drinking water quality', Wat. Res., 23, 1313-1322.
Voss, L., Button, K.S. and Tuovinen, O.H. (1985) '*Legionella pneumophila* in a metropolitan water distribution system', Environ. Technol. Letters, 6, 429-438.

BIOFOULING ON MEMBRANES - A SHORT REVIEW

H.-C. FLEMMING, G. SCHAULE and R. McDONOGH
c/o *Institut für Siedlungswasserbau, Wassergüte- und Abfallwirtschaft der Universität Stuttgart, Bandtäle 1, D-7000 Stuttgart 80, Germany*

1. Introduction

"Fouling" is referred to as the unwanted deposition of material from the bulk water phase on surfaces, such as membranes. This term has been adopted from heat exchanger technology (Epstein, 1981; Characklis, 1990). For membrane technology, the most important types of fouling include:

* crystalline fouling ("scaling", deposition of minerals due to excess of the solution product)
* organic fouling (deposition of oil, grease, lipids etc.)
* particle fouling (deposition of clay, silt, humic substances, debris etc.)
* colloidal fouling (silica, humic acids)
* biofouling (adhesion and accumulation of microorganisms)

While the first four types of fouling can be controlled by chemical pretreatment of the water, addition of conditioning agents or by filtration, microorganisms are particles which can multiply at the expense of the nutrients in the water. Thus, a reduction of the number of cells does not help very much, because they will regrow to the old extent if the concentration of nutrients is not drastically reduced. Biofouling usually does not occur in a spectacular way but is more "sneaking" because of the gradual accumulation of the biological deposit, called "biofilm". *Biofouling is a biofilm problem.* A biofilm is the result of adhesion and growth of bacteria on an interface. There is almost no surface which cannot be colonized or is already colonized by microorganisms. Thus, biofilms are ubiquitous. Especially in low nutrient environments, adhesion to surfaces is considered to be a natural strategy of bacteria in order to survive (Marshall, 1985).

Biofilms usually exhibit a more or less slimy consistency and represent a highly hydrated gel matrix in which the microorganisms and eventual particles are immobilized. Biofilms consist mainly of the following components:

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 487–497.

* Water (50-95 % of wet weight)
* extracellular polymer substances (EPS; "slime", "organic glue"), excreted by the microorganisms (50-95 % of dry weight)
* Microorganisms
* entrapped particles
* dissolved substances (the gel matrix acts as a molecular sieve and can accumulate ions and organic molecules from water phase)

The composition may vary greatly, but the slimy consistency and the high water content are characteristic; the microorganisms themselves may contribute only to a minor part to the total mass of the biofilm. Biofilms in technical systems range between 5 and >500 μm in thickness. Biofilms are structured in a laminar way. Aerobic microorganisms live at the surface of the film and consume oxygen. In a depth of 50-100 μm they tend to be completely anaerobic because of the oxygen consumption of the microorganisms in the surface layer of the biofilm. This means that even in an aerobic system, biofilms can create anaerobic sites, resulting in reducing chemical conditions and the growth of anaerobic microorganisms such as sulfate reducers. In the presence of ferrous ions, iron sulphide is formed which results in black coloured deposits.

2. Effects of biofouling on membrane processes

On a separation membrane, the biofilm matrix represents a secondary membrane which participates in the separation process. Due to the diffusion resistance of the gel matrix there is no convectional mass transport possible. This tends to increase the concentration polarization on the membrane, leading to scaling problems. The biofouling layer by itself increases the membrane Δ-P, i.e. the transmembrane pressure difference between concentrate and permeate and, eventually, the material can be attacked by microbial activity, as has been observed with cellulose acetate membranes. In spiral wound membrane modules, the biofilm can overgrow the spacer and, thus, cancel its hydrodynamic effect. Additionally, biofilms lead to an increase of friction resistance for water because of their rough and viscoelastic surfaces (Christensen and Characklis, 1990). These effects can cause a severe pressure drop in flowing systems. Case histories have been collected by Ridgway (1988) and Flemming (1992). The consequences of biofouling on membranes have been summarized as:

* *Increased membrane resistance by the biofilm*:
  - Decrease of permeate production;
  - Increase of energy consumption;
  - Increase of membrane Δ-P.
* *Formation of a gel phase between water and membrane surface*:
  - Convectional transport next to the membrane surface is inhibited;
  - Increase of concentration polarization due to lack of tangential flow;
  - Decrease of salt rejection.

* *Damage to plant and product*:
  - Eventual microbial attack on membranes (e.g., on cellulose acetate)
  - Microbial contamination of the permeate
  - Decrease of module lifetime (increased number of cleanings)
* *Increased costs*:
  - Production loss (less permeate);
  - Product quality loss (higher salt content, microbial contamination);
  - Increased energy costs;
  - Increased cleaning demand;
  - Increased replacement costs.

3. Development of biofilms

Three phases characterize the process of biofilm accumulation: i) the induction phase with primary colonization (important for stimulation or prevention of biofilm development), ii) the logarithmic growth phase and iii) the plateau phase, when a steady state develops between accumulation and desorption (important for stability and removal of biofilms). Biofilm accumulation can roughly be described in a sigmoidal curve as shown in Fig. 1 (after Characklis, 1990):

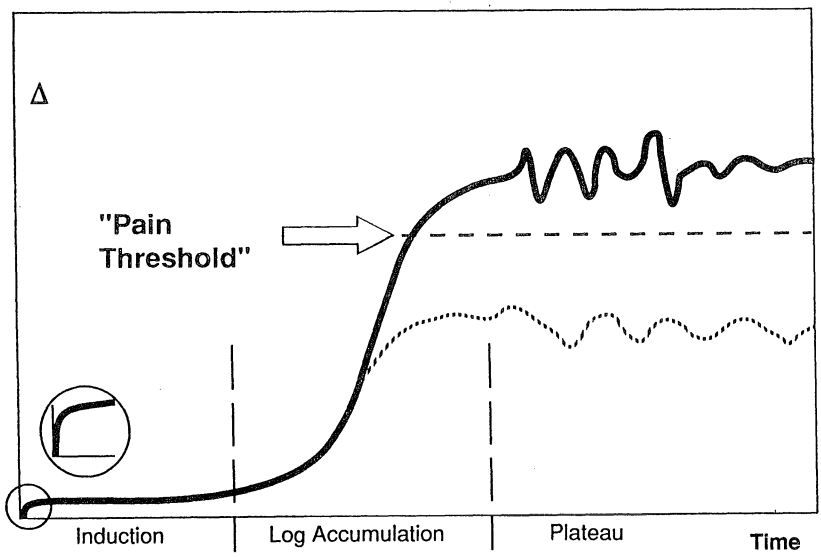


Fig. 1: Time dependent development of biofilm accumulation, after Characklis (1990). Δ = biofilm growth parameter (thickness, weight etc.).

Actual experimental data illustrating the inset in Fig. 1 is given in Fig. 2. It shows how rapidly primary colonization occurs to reach a primary plateau (Flemming and Schaule, 1988). This has been observed in batch experiments as well as in a dynamic test cell with reverse osmosis (RO) membranes and tap water (Flemming et al., 1992). After one day of operation with clean tap water, the membrane is colonized by approx. 5 x 10^7 cells/cm^2, resulting in a complete coverage and a flux decline of

x 10^7 cells/cm^2, resulting in a complete coverage of the membrane surface and a flux decline of about 30 % (Figs. 3-5).

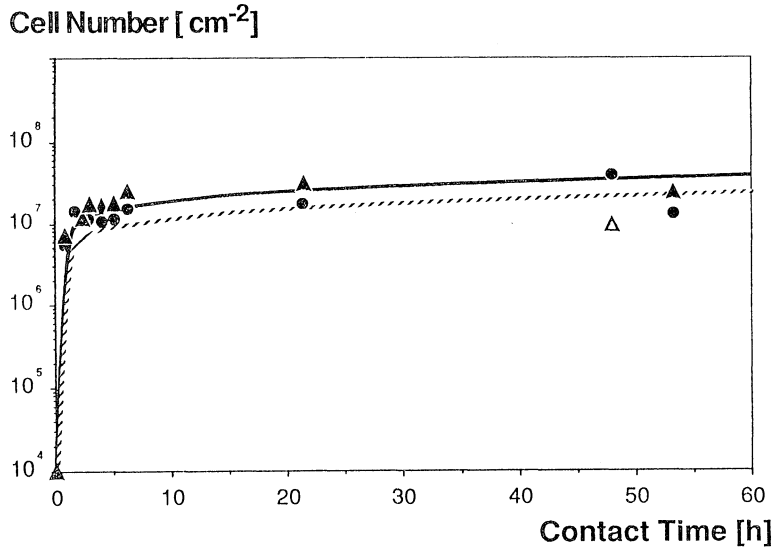


Fig. 2: Primary colonization of polysulfone membrane material. o = living cells, Δ = dead cells (Flemming and Schaule, 1988, modif.).

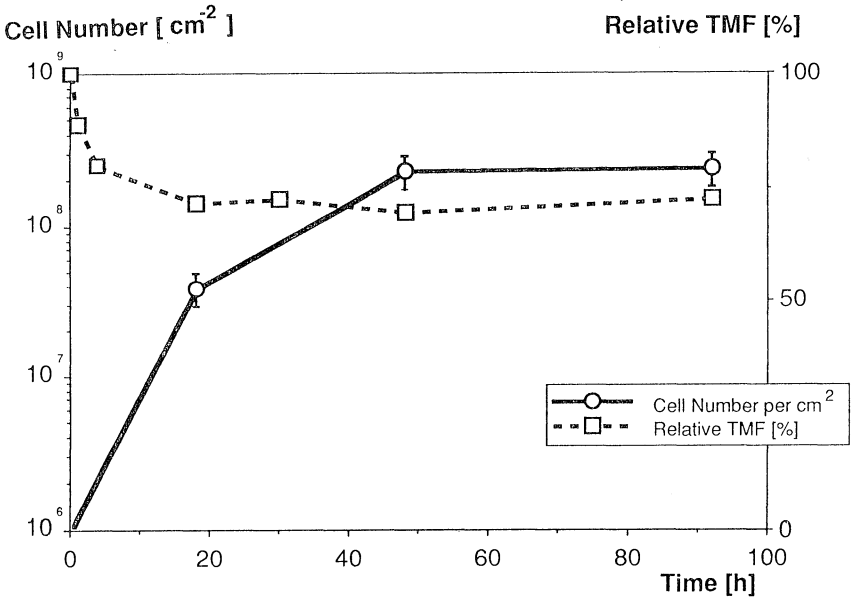


Fig. 3: Colonization (cells per cm^2) of a polyamide membrane (FT 30) during operation with clean tap water. Flux decline is also shown as percentage of pure water transmembrane flux (TMF [%]) (Flemming et al., 1992).

The cells originally came from the microflora of the raw water, which contained 10^5 cells/ml, whereas standard drinking water methods led to numbers of colony forming units (CFU) of 8-10 per ml only. This arises from the fact that colonization methods detect only 1-10% of the microorganisms present in a water sample. Many natural organisms cannot be cultured on standard nutrients.

These observations trigger the assumption that every operating RO-membrane will most probably carry at least a thin biofilm on the membrane surfaces, providing the potential for further accumulation, if the conditions allow. This primary biofilm will exhibit its own characteristic permeation resistance.

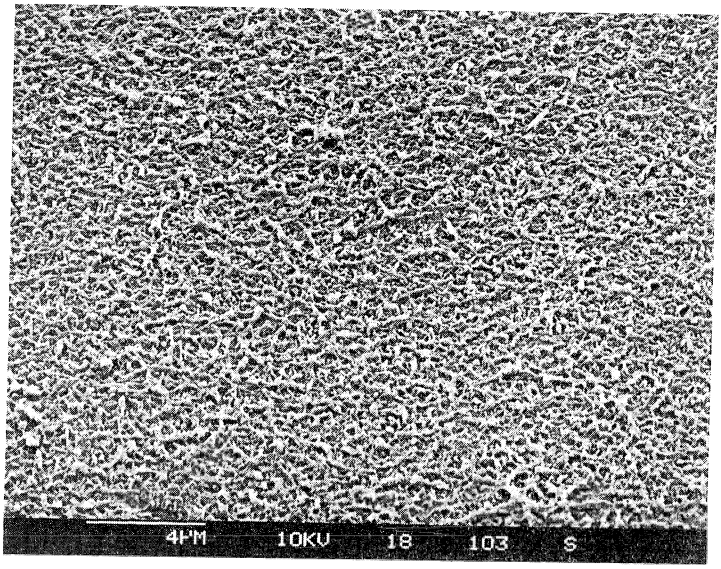


Fig. 4: Surface of a clean polyamide membrane as used in experiments shown in Fig. 3.

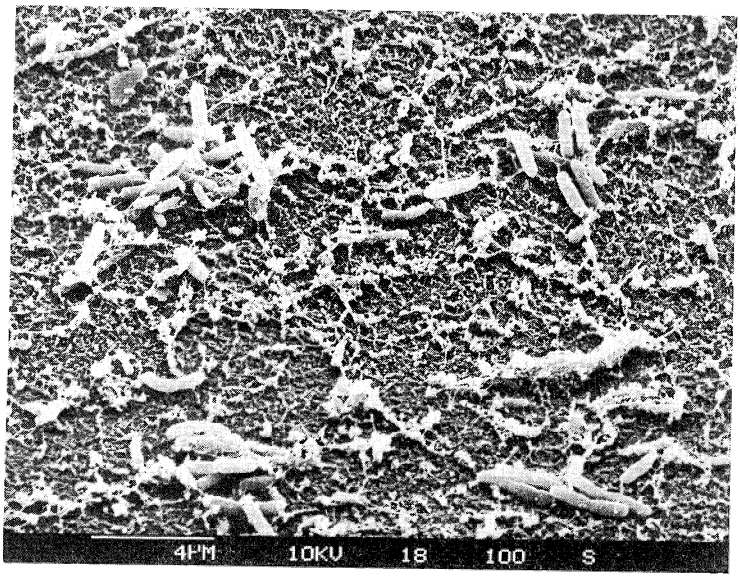


Fig. 5: Surface of the same membrane after 24 h of operation with clean tap water as feed.

However, the extent of accumulation may stay below the "pain threshold" of any given target value. This value will take in account a "fouling factor" which is caused among others by the primary fouling layer. Only when the limit value is exceeded, e.g. a flux decline of more than 30 %, would the operator of the plant feel the necessity to do something to counter symptoms like pressure drop or flux decline, and then respond to the "pain". It is very important to keep in mind that in the latter case biofouling had not just started at the time when the parameters alerted the operator but long before. The underlying problem is the biofilm already present, and something has happened which interfered with the previous equilibrium and lifted the plateau above a certain level of interference.

4. Detection of biofouling

In an operating plant, bacterial growth will not occur significantly in the water phase while it passes the plant. The residence time of the water is too short (multiplication times are approximately 2 h or more under the given conditions). What grows is the biofilm on all surfaces of the system. This includes membranes, pipe walls, cartridge filters, valves, O-rings and all other materials (even if they are only in contact with humid air). These biofilms eventually seed cells into the water phase, and these are measured by the CFU counts taken from water samples. The first answer to biological problems is always sampling of the water phase. *However, there is no correlation between cell numbers in the water phase and the location and extent of biofilms*. Cells can be eroded by shear forces, they can leave the biofilm as swarmer cells or eventually, parts of the biofilm can be sloughed off, giving rise to substantial increases in cell numbers in the bulk. All these events occur randomly. Biofouling arises from surfaces and cannot be treated as a problem of the water phase only.

It must be considered that even in high purity water (18 MOhm) systems biofilms have been observed (Paterson et al., 1991; Mittelman, 1991). The cell density in the water phase was below 1-10 CFU/ml, while the cell density in a biofilm was between 10^7 and 10^{11} cells/ml biofilm mass. On the other hand, high cell numbers in the bulk may not necessarily indicate thick biofilms. Thus, samples representative for biofouling have to be taken from surfaces. Otherwise one only gets those cells which have randomly been released by the biofilm. This means, that *the microbiological data as collected from practically every plant do not help to localize* the biological problem. In practice, piles of data have been collected, which give *no* meaningful information.

It is important to take the complete system into consideration. As a consequence, not only the membranes but also other surfaces have to be observed and can be used for detection of biofouling.

Usually, the diagnosis "biofouling" is given when there is no other explanation for the problem. This is fair enough, because most of the membrane systems suffer from biofilms. However, data which could *prove* the existence of biofilms on membranes

can only be given by surface samples and in most cases must be gained by destructive analysis. In practice, this can be performed with sacrificial elements, test coupons, or by an "autopsy" of irreversibly blocked modules (Ridgway et al., 1984; Flemming u. Schaule, 1989). The analysis of the deposit should include measurement of a combination of the following parameters (Flemming, 1992), to confirm the presence of a biofilm:

* Water content;
* Organic matter (total organic carbon [TOC], incineration residue);
* Microscopic counts (Epifluorescence or others);
* Plate counts (cultivation methods using appropriate media; many biofilm bacteria are not culturable on nutrient-rich agar!);
* Sulfate reducers, slime formers;
* Presence of protein, carbohydrates, ATP, muramic acid, phospholipids;
* Activity of selected enzymes which can be easily measured.

5. Sanitization

The most common response to biological problems in a technical system is the application of a biocide (e.g., Ridgway and Safarik, 1991). Fig. 2 shows, however, that the problem is not solved simply by killing the microorganisms. There are always some that can attach to surfaces even when they are dead, thus, providing both substrate and readily colonizable surfaces for subsequent cells imported by the raw water. Dead biofilms still represent a problem; additionally, biofilm organisms are much more difficult to kill than suspended cells, because the biofilm matrix protects its inhabitants (LeChevallier et al., 1988). It seems to be quite useless to "sterilize" a technical system which is otherwise run in an unsterile mode of operation. Thus, the use of biocides at least has to be completed with effective cleaning measures. It seems much more important to remove the biomass rather than killing it and leaving it where it was. However, that is eactly what biocides such as formaldehyde do, which having been applied in many RO plants as a "cleaner". It has been used as a fixing agent in microscopy because it links protein chains and binds biological material together and to the support.

Do biocides help to keep the plateau level low, *c.f.* Fig. 1? Miller (1982) and Bott (1990) have investigated the problem using biofilm accumulation on heat exchanger surfaces as an example. They used a flow-through system where bacteria came from a fermenter and could attach to a metal surface. After biofilm accumulation had reached a plateau, they shut down the supply of new bacteria in the water phase, keeping the nutrients at the same level as before. The level of the plateau was not affected. However, when they decreased the nutrient concentration (without excluding the bacteria) instead, the plateau was significantly lower. What does this say for biofouling in a membrane system? Disinfection of the feed water - at its best - is the attempt to shut down the supply of new bacteria in the water phase. However,

the level of the plateau phase is almost independent from the number of cells in the bulk liquid. Thus, once the biofilm is established (usually after only short time), it is much more dependent on *nutrient supply and shear forces than on cells in the feed.*

The crucial point is: to remove the deposit, or, at least, to curb the extent of its accumulation. In this case, one has to overcome the physical strength and integrity of the biofilm matrix. The target is mainly the gel network of slime substances (EPS), which is frequently supported by abiotic filamentous matter. Incomplete removal of the deposit is the main reason for rapid aftergrowth and for the sawtooth shaped curve of performance over periods of many cleaning cycles. Fig. 6 shows a scanning electron micrograph of a biofouled membrane. The deposit is the net result of biofilm accumulation, having survived many cleaning cycles. Fig. 7 shows the same membrane after external cleaning with 1 % Ultrasil 53 for 24 h and still carries substantial amounts of the deposit after this extreme treatment.

Figs. 6 and 7 illustrate another problem in the struggle against biofouling: the effectivity control. The success of a cleaning regime is always evaluated by the process parameters, which have to meet some standards, specific for a given system. In some cases, cell numbers in the water phase are measured, however, without giving valuable data. As discussed before, these numbers do not indicate the extent or site of biofilms. Thus, we still have a biofilm, only it does not pass through the "pain threshold" (Fig. 1). This can be termed a "waiting biofilm", which will accumulate because of the nutrients in the raw water and not because of the adsorption of the suspended cells. If conditions allow, it will form a new tooth of the sawtooth curve.

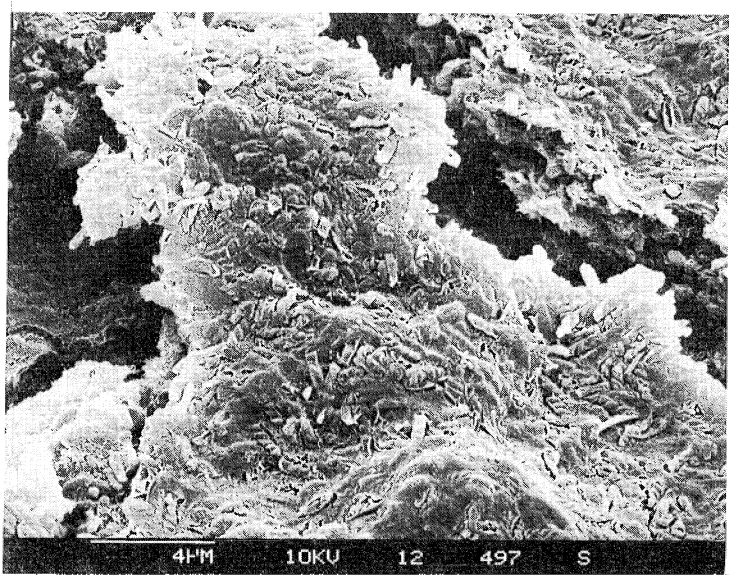


Fig. 6: Irreversibly biofouled FT 30 membrane (river water purification); note that the membrane has undergone many cleaning cycles; bacteria are embedded in the slime matrix

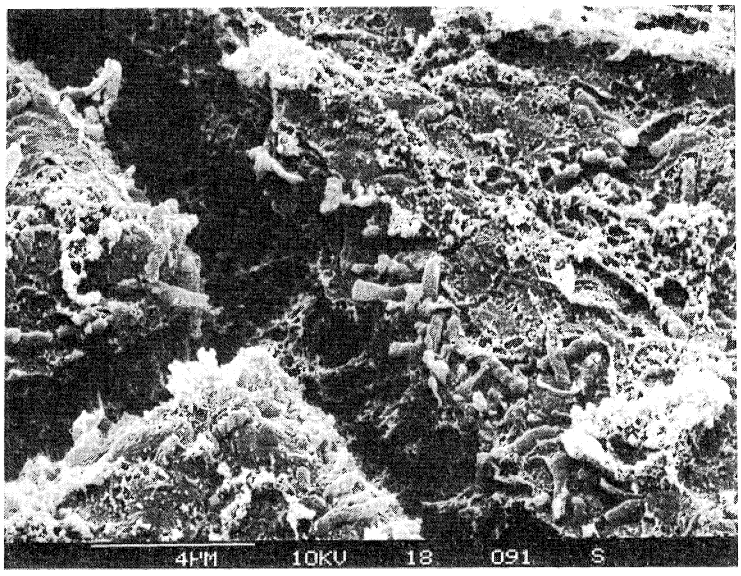


Fig. 7: Membrane material from Fig. 6 after prolonged (24 h) cleaning with Ultrasil 53. Note the considerable deposit which still remains. This will give rise to rapid aftergrowth

Highlighted by this background, it seems very recommendable to follow a two step sanitization strategy:

- the *first step* focuses on weakening of the biofilm matrix and is performed in most cases by chemical methods. These include oxidizing agents, as far as they can be applied in a given environment (e.g. some membrane polymers will not tolerate them), and combinations of biodispersants, surfactants and enzymes.

- the *second step* focuses on removal of the deposit from the system and is usually performed by physical methods - such as shear forces, mechanical methods, application of ultrasonic energy etc.

The effectiveness of each step has to be validated in a representative manner, an exercise which is missing in most cases. This is particularly irrational, because the cleaning regime has to protect a very costly system. Effectiveness control helps to optimize the the cleaning regime. Emphasis must shift from killing to removing the biomass.

6. Permeation properties of biofilms

It has been stressed that killing of the biomass is not of great help in a fouled system. If the application of a biocide leads to transient success, maybe that this is not due to the killing of the microorganisms but due to the changes in the biofilm matrix, caused by the chemical reaction of the biocide with the EPS. Thus, the biomass has not been removed but the permeation properties of the biofilm - a secondary membrane on the separation membrane - have been changed.

Flemming et al. (1992) present a preliminary analysis of this. The biofilm has been modelled by a hydrogel of 2 % Agar in water. The hydrogel was placed on a filter membrane and water was pressed through the membrane. After the permeate flux had reached a constant value, a commercial cleaner was added and passed through the hydrogel and the membrane, followed by clean water. Fig. 8 shows the development of both permeate rate (permeability) and filter cake height.

The result clearly shows that the permeation rate has been improved by almost one order of magnitude without any change in the thickness of the hydrogel. The effect was maintained after the cleaner had been washed out.

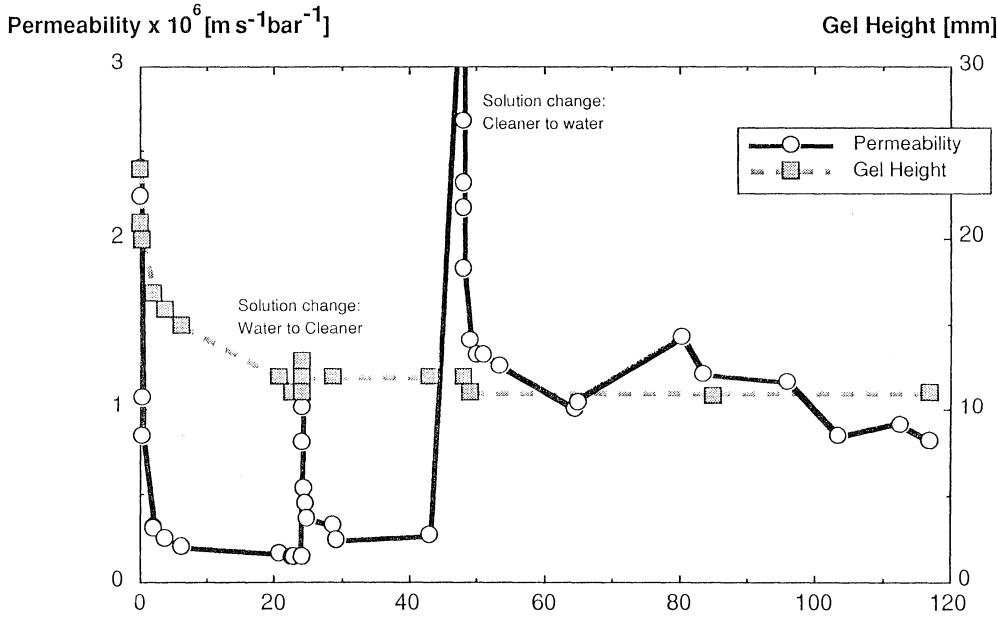


Fig. 8: Change with time [h] of the permeation rate (permeability) of a hydrogel after application of a commercial cleaner; note that the height of the filter cake remained constant (Flemming et al., 1992)

These data show that an improvement of permeate flux by cleaners, applied to a hydrogel which acts as a secondary membrane, can be due to chemical alterations of the matrix structure. This may be true for biofilms as well, and it will be investigated in further experiments. If this is the case, the "success" of a cleaning measure in practice may at least partly be due to an improvement of the permeation properties of the fouling layer instead of the removal of the material. Perhaps, a substantial part of the improvement of performance parameters after cleaning is due to this effect.

Acknowledgements

The authors wish to thank the Willy-Hager-Stiftung, Stuttgart, for its financial support. The excellent assistance of Hanna Rentschler is gratefully acknowledged.

7. References

Bott, T.R. (1990): Bio-Fouling. In: Bohnet, M. (ed.): Fouling of heat exchanger surfaces. VDI publication, P.O.Box 1139, D-4000 Düsseldorf 1, pp 5.1-5.20

Characklis, W.G. (1990): Microbial fouling. In: Characklis, W.G. and K.C. Marshall (eds.): Biofilms. John Wiley, New York, 523-584.

Christensen, B.E. and W.G. Characklis (1990): Physical and chemical properties of biofilms. In: Characklis, W.G and Marshall, K.C. (eds.): Biofilms. John Wiley, New York; 93-130

Epstein, N. (1981): Fouling: technical aspects. In: E.F.C. Somerscales and J.G. Knudsen (eds.): Fouling of heat transfer equipment. Hemisphere, Washington; 31-53

Flemming, H.C. and G. Schaule (1988): Biofouling on membranes - a microbiological approach. Desaliation **70**, 95-119

Flemming, H.C. and G. Schaule (1989): Biofouling auf Umkehrosmose- und Ultrafiltrationsmembranen. Teil II: Analyse und Entfernung des Belages. Vom Wasser 73, 287-301

Flemming, H.C. (1992): Mechanistic aspects of reverse osmosis membrane biofouling and prevention. In: Z. Amjad (ed.): Reverse osmosis technology. Vol. 1. Marcel Dekker, New York; in press

Flemming, H.C., G. Schaule and R. McDonogh (1992): How do membrane parameters respond to initial biofouling? Vom Wasser **76**, in press

LeChevallier, M. W., C. D. Cawthon and R. G. Lee. 1988. Inactivation of biofilm bacteria. Appl. Environ. Microbiol. **54**; 2492-2499.

Marshall, K.C. (1985): Bacterial adhesion in oligotrophic habitats. Microb. Sci. 2, 321-326

Miller, P.C. (1982): Biological fouling film formation and destruction. PhD Thesis, University of Birmingham

Mittelman, M.W. (1991): Bacterial growth and biofouling control in purified water systems. In: H.C. Flemming and G.G. Geesey (eds.): Biofouling and biocorrosion in industrial water systems. Springer, Heidelberg, New York; 133-154

Paterson, M.K., G.R. Husted, A. Rutkowski and D.C. Mayette (1991): Isolation, identification and microscopic properties of biofilms colonizing large-volume UPW distribution systems. Ultrapure Water, May/June 1991, 18-24

Ridgway, H. F., C. A. Justice, C. Whittaker, D. G. Argo and B. H. Olson (1984): Biofilm fouling of RO membranes-its nature and effect on treatment of water for reuse. J. Amer. Water Works Assoc. **79**, 94-102.

Ridgway, H.F. (1988): Microbial Adhesion and Biofouling of Reverse Osmosis Membranes. In B. S. Parekh (ed.): Reverse Osmosis Technology: Applications for High-Purity Water Production. Marcel Dekker, New York, New York; 429-481.

Ridgway, H.F. and J. Safarik (1991): Biofouling on reverse osmosis membranes. In: H.C. Flemming and G.G. Geesey (eds.): Biofouling and biocorrosion in industrial waters systems. Springer, Heidelberg, New York; 81-111

BIOFOULING IN HEAT EXCHANGERS

L. F. MELO and M. M. PINHEIRO
University of Minho - Biological Engineering
4700 Braga, Portugal

1. Introduction

Heat exchangers are used to transfer heat from a hot fluid to a cold fluid. In general, the two fluids flow through appropriate channels (tubes, spaces between parallel plates or between tubes) within the equipment. Solid surfaces made of metal or, less often, of some polymer (teflon, e.g.) separate the fluids.

Although there is a wide variety of configurations, the most well known is probably the shell-and-tube heat exchanger, where one fluid circulates within the tubes and the other flows outside the tubes, in the shell. Heat exchangers of this type are frequently used in cooling water systems. In condensers, the water flows within the tubes and the hot vapour circulates in the shell.

Cold water is available from natural sources (rivers, bore holes, etc.) which contain microbial species, organic substances and inorganic particles. By receiving heat from the hot fluid, the cold water temperature reaches 30-40 °C in some parts of the exchanger. This falls within the range of optimum temperatures for microbial growth, facilitating the formation of a biofilm attached to the solid surfaces that separate the two fluids. The performance of the heat exchanger is disturbed by this unwanted phenomena (biofouling), because it increases the thermal resistance between the fluids and the pressure drop of the flowing water.

Sometimes, the mass flow rates of water in these cooling systems are very large (such is the case of power plant condensers) and, as a consequence, the use of certain anti-biofouling techniques (biocides, additives) is very expensive.

2. Factors affecting biofilm behaviour in heat exchangers

Since biofouling is the result of the accumulation and survival of microorganisms on solid surfaces, the environmental conditions will be of fundamental importance on the overall process of biofilm formation. Obviously, the latter depends also on the microbial species present in the water, mainly on their ability to adhere to the surface and on their biological activity in the film. The influence of operating variables such as

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 499–509.

the fluid velocity, temperature and composition, as well as of the surface characteristics has been studied and can to a certain extent be controlled in the design and operation of heat transfer equipment.

2.1. SURFACE CHARACTERISTICS

Surface characteristics are specially relevant during the build-up of the first layers of biofilm. For example, surface roughness plays a significant role in the transport and adsorption of the first macromolecules and microbial cells to the surfaces. In fact, apart from increasing the available interfacial area, a rough surface enhances mass transfer coefficients and allows cells to "anchor" on its microirregularities where they are better protected from possible desorption (Characklis, 1990).

It can be said that polished surfaces may enlarge the induction period of biofouling formation which is of interest in industrial operations, but the final amount of deposit is not significantly affected by this surface caharacteristic (Lund and Sandu, 1981).

Regardless of the surface roughness, the attachment of living particles is energetically favourable if the change in the free energy during the process of adhesion is negative. In that sense, surface coatings with smaller surface tensions, or surface tension depressants, or detergents in dilute nutrient solutions can be used to delay the attachment of the first layers (Baier, 1979). Materials with different critical surface tensions were utilized by Dexter (1979) to investigate the differences in the attachment of living cells. He concluded that for a range of surface tensions that include polymers there was a low adhesion capacity, but on the other hand most heat exchanger materials (metals) possess surface tensions that allow an easy adsorption of the first layers of biofilms.

In spite of metallic surfaces being energetically favourable to the establishment of the first cells, the chemical composition may interfer on the cellular metabolism, including the production of the exopolymers. Results obtained by Duddridge *et al.* (1981) reveal brass as the metallic surface with lower levels of bacteria attachment, as compared with stainless steel or aluminium. Similar indications were found by Vieira *et al.* (1992a) when counting the number of attached cells of *Pseudomonas fluorescens* on brass, copper and aluminium surfaces after a few hours of exposure. Aluminium was the easiest fouled surface, followed by copper and brass. Copper ions have been claimed as microbial metabolic inhibitors and zinc ions as delayers of cell adhesion. However, Garey (1979) compared the accumulation of biofilms on copper alloys and on stainless steel or titanium surfaces, and the only difference was the extended induction period for copper alloys. The total amount of film obtained in each case was approximately the same.

The effect of Zn, Cu and Al ions on *Pseudomonas fluorescens* growth rate was investigated by Vieira *et al.* (1992b) in shake flask cultures, indicating a long lag phase in the presence of Zn ions and a significant reduction in the growth rate of the bacteria when copper was added (Figure 1). Neverthless, there are references (Bush, 1971; Pinheiro *et al.*, 1988) of large biofilm accumulation of this bacterium on copper surfaces. It is thought that the microrganisms are able to overcome the inhibiting effects

of Cu ions near a surface by producing large quantities of biopolymers (Eaton *et al.*, 1980).

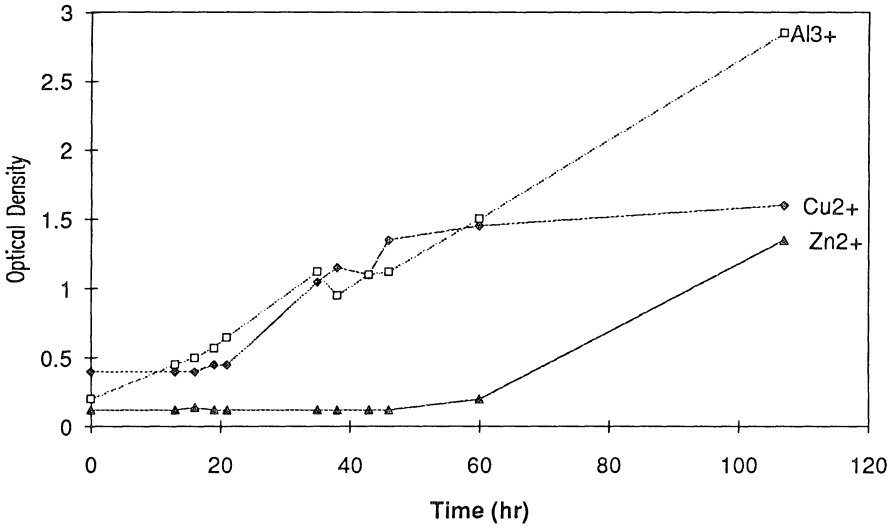


Figure 1 - Effect of dissolved metallic ions on the growth rate of *Pseudomonas fluorescens*

2.2. FLUID VELOCITY

Fluid velocity is a critical factor influencing the build up of biofilms. Lower velocities facilitate the adhesion of cells, but, at the same time, the growth rate may become limited by the slower substrate transport to the attached cells. In the high velocity region, shear stress has a great effect on the total accumulation of biofilms. In fact, results obtained by Duddridge *et al.* (1982) and by Pinheiro *et al.* (1988) show a drastic reduction in the amount of biofilm when fluid velocity is increased. This does not necessarily imply a reduction in microbial growth rate. For example, Sanders (1964) reported increased values of oxygen uptake in thin biofilms formed under high fluid velocities, which seems to indicate a higher microbial activity. In the case of Duddridge *et al.* (1982) and of Pinheiro *et al.* (1988), biofilm thickness was controlled by the effects of fluid shear stress.

Microbial activity in biofilms is extremely dependent on the availability of nutrients near the cells, i.e., on the effectiveness of the diffusion process throughout the film. However, mass transfer rates depend not only on the film thickness but also on its structure. Mass transfer measurements carried out in biofilms formed under different fluid velocities, using the same microorganism as a contaminant, showed that as the Reynolds number increased, the mass transfer coefficient in the final biofilm decreased (Vieira *et al.*, 1992c). Since the film is thinner for higher Reynolds numbers (Bott and Pinheiro, 1977), the reduction in the rate of mass transfer throughout the biofilm is probably due to changes in its spatial structure caused by the hydrodynamic conditions.

Greater velocities originate more compact biofilms where the diffusion of substrate will be more difficult. Although the biofilm compactness was not measured, densities of dry *Pseudomonas fluorescens* biofilms were found to increase with the flow velocity (Pinheiro *et al.*, 1988), this seeming to suggest a more complex film structure for higher velocities.

2.3. NATURE OF THE FLUID

2.3.1.Substrate concentration

Generally, it can be said that the extent of biofilm accumulation increases with substrate loading.

High concentrations of substrate favour the growth rate of microorganisms, increasing the number of cells in the flowing fluid which eventually will adhere to the solid surfaces. Trulear and Characklis (1982) report larger cell concentrations in biofilms when high loadings of substrate are present.

Biofilm densities are also reported to increase with substrate concentration (Characklis et al., 1988). Since nutrients must diffuse through the film in order to maintain the biological activity of the attached cells, there will be probably a limit beyhond which cell concentration in the biofilm will not increase even if substrate concentration recahes very high values (Characklis, 1980). If, for example, oxygen is the limiting substrate, anaerobic zones may develop in the inner parts of the film causing in some cases the sloughing off of the biolayer (Howell and Atkinson, 1976).

The effect of the reduction of substrate concentration on a previously formed biofilm is illustrated in Figure 2. A drastic decrease in the asymptotic value of the heat transfer resistance was observed after the exclusion of the substrate (Oliveira *et al.*, 1992). This was probably due to the decrease in the production of extracellular polyssacharides, making the film more vulnerable to the fluid shear forces.

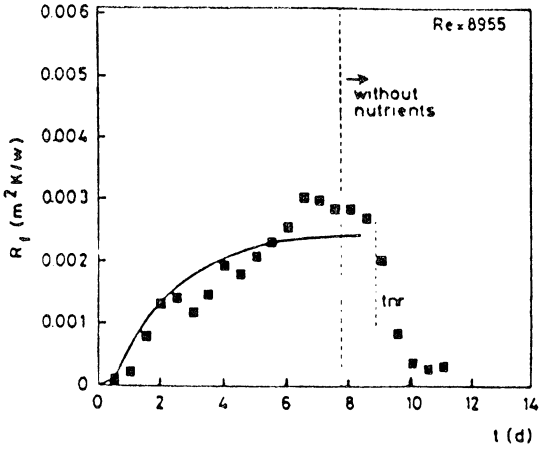


Figure 2 - Effect of the reduction of substrate concentration on the behaviour of a previously formed biofilm

2.3.2.Inorganic particles

Untreated water contains often significant levels of inorganic substances that may induce synergistic effects on the build up of biofilms. For instance, clay particles present in river water seem to promote changes in the amount and, possibly, the structure of the films, such as discussed elsewhere in this book (Bott and Melo, 1992).

It is worth noting that if the concentration of suspended solids is very high, it can produce a scouring effect on the surface and reduce biofilm accumulation (Battaglia *et al.*, 1981).

2.3.3.Fluid and surfaces temperatures

Growth and reproduction of microorganisms are greatly influenced by temperature. Biofilm development has been related to the bulk temperature as well. In general, the problems caused by biofouling in industrial cooling water systems deserve more attention during summer than during winter periods due to the warmer inlet temperatures of the water.

For example, films formed by *E. coli* in a heat exchange apparatus almost doubled their thickness when the fluid temperature varied from 30 °C to 35 °C (Bott and Pinheiro, 1977) - the optimum temperature for that species is 37 °C.

Stathopoulos (1987) studied biofouling caused by a mixed population under a range of temperatures from 15 °C to 40 °C. The film thickness and mass increased substantially with temperature until the latter reached 45 °C. Then the trend was reverted.

Sometimes, the surface temperature can have a greater influence than the fluid temperature. In fact, results obtained by McCaughey *et al.*(1987) show higher accumulation (mass and number of cells) on heated than on non-heated tube surfaces for the same bulk temperature. On the other hand, Hatori and Hatori (1981) found a reduction in biofilm thickness when the surface temperature varied from 30 °C to 50 °C, and argued that the higher surface temperatures could be responsible for a lower production of exopolymers by the cells affecting their adhesion mechanism.

3. Effects of biofouling on heat exchanger design and performance

3.1. ECONOMIC ASPECTS

The economic penalties related to the occurence of biofuling in heat exchangers include the costs of : 1) extra heat transfer area (see section 3.2.); 2) water treatment and additives; 3) energy consumption due to increased pressure drop (section 3.3.) and heat transfer inefficiency (section 3.2.); 4) production losses caused by unwanted plant shutdowns, maintenance and cleaning. According to Thackery (1979), the contribution of each of these four items to the total fouling cost ranges between 20% and 30%.

3.2. EFFECTS ON HEAT TRANSFER

The heat transfer rate bewteen a hot fluid h and a cold fluid c in a heat exchanger is given by the fundamental equation :

$$q = F\,U\,A\,(T_h - T_c)_{mean} \qquad (1)$$

where q is the heat transfer rate (W), U the overall heat transfer coefficient (W/m^2K), A the area of the surface that separates the two fluids (m^2) and $(T_h - T_c)_{mean}$ the mean temperature difference between the two fluids in the heat exchanger. F is a corrective factor depending on the type of heat exchanger and on the type of flow arrangements (usually between 0.75 and 1.0).

The overall heat transfer coefficient is a measure of the effectiveness of the heat transfer mechanisms. If the two fluids and the wall between them let the heat flow "easily", U will have a large value. If the fluids and/or the wall are poor heat transfer media, then the value of U will be much smaller. This value is calculated, for a plane and clean wall, by :

$$\frac{1}{U_0} = \frac{1}{h_h} + \frac{1}{h_c} + \frac{1}{k_w} \qquad (2)$$

where U_0 is the overall coefficient when the surface is clean, h_h and h_c are the convective heat transfer coefficients for fluids h and c, x_w and k_w are the thickness and the thermal conductivity of the wall, respectively. Each fraction in Equation 2 represents a thermal resistance and the overall resistance is the sum of the resistances offered by each medium (fluids and wall) to the flow of heat. When a deposit, such as a biofilm, builds up on the solid surface, it adds a new resistance (R_f) to the flow of heat and causes a reduction in the overall coefficient :

$$\frac{1}{U} = \frac{1}{U_0} + R_f \qquad (3)$$

U ($< U_0$) represents the overall coefficient with a "dirty" or fouled surface. R_f, the thermal resistance of the biolayer, is often called the "fouling factor". The decrease in U reduces the the heat transfer rate bewteen the two fluids and causes poor performance of the heat exchanger (for example, the outlet temperature of the hot fluid will be higher than it should be). To circumvent this problem, an "expected" value of the fouling factor is introduced in the design of the heat exchanger. The result is that the heat transfer area (A) will be greater than in the case of a clean wall, leading to an overdesigned and more costly piece of equipment. Frequently, values of U are specified by the future user of the exchanger, based on previous experience or on recommended

values given by TEMA (Tubular Exchanger Manufacturers Association, USA).

In a water cooled condenser, the clean overall coefficient may be considered to fall typically within the range of 1000-4000 W/m^2K, and the fouling factor is in the order of 0.0002 m^2K/W (Chenoweth, 1988), the latter including biofilm thermal resistance. Therefore, the overall coefficient in fouled conditions will be between 830 and 2200 W/m^2K, implying an increase of 20% to 45% in the surface area needed.

3.3. EFFECTS ON PRESSURE DROP

In general, the attached films are rougher than the initial clean surface. Sometimes, when the thickness is large as compared to the tube diameter, they may also cause partial obstruction in the flow passage. In both cases, the pressure drop in the heat exchanger will increase, leading to higher pumping costs. The increase in pressure drop is a rather economic means of detecting the buildup of deposits in industrial equipment.

3.4. CORROSION EFFECTS

Interactions between corrosion and biofouling occur very often. Some of the bacteria detected in biological deposits produce sulfur compounds or acids that attack the metallic surfaces where the biofilm is attached (microbial induced corrosion is reviewed in another text in this book - Chamberlain, 1992). If appropriate surface materials or corrosion inhibitors are not used, heat exchanger parts (tubes, tube sheets, etc.) may have to be replaced frequently as a consequence of their deterioration.

3.5. PROBLEMS ASSOCIATED WITH HEAT EXCHANGER OVERSIZING

As mentioned in section 3.2., oversized heat exchangers are designed to compensate for the incresed thermal resistance caused by the attached layer. In a tubular heat exchanger, a larger heat transfer area means that (a) the tubes will have to be longer; or (b) the tubes should have a larger diameter; or (c) a higher number of tubes is needed. If the tube length is limited by the available space or the allowable pressure drop, the use of procedures (b) and (c) can lead to lower fluid velocities in the tubes (for a given flow rate), which tend to enhance fouling (section 2.2.). Having this in mind, a good design strategy should be able to overcome the problem.

There is another question that may be more difficult to solve. Whereas the heat exchangers are calculated assuming steady state operation, the buildup of fouling layers is a transient phenomena. The exchanger is designed to yield a fixed heat transfer rate with a completely developed deposit. Consequently, in the first days/weeks of operation, when the surfaces are still reasonably clean, the heat flux will be higher than predicted, and this will originate higher temperatures that can favour, in many instances, the rapid development of fouling in certain zones of the equipment. To compensate for this effect, cooling water flow rates are sometimes reduced by the operator, but this will tend to cause more deposition in the tubes.

3.6. SOME ASPECTS OF BIOFOULING PREVENTION IN HEAT EXCHANGERS

Several measures can be taken to reduce the occurence and impact of biofouling in heat exchangers. The use of biocides is one of them and is discussed in another paper in this volume (Bott, 1992).

The choice of suitable materials is an important factor , but it can be limited in industrial equipment by economic and process reasons. In principle, surface materials should have low surface energies, high thermal conductivities, be smooth, resist process temperatures and pressures, and resist corrosion. Titanium, for instance, is a rather expensive material used in heat exchangers cooled by sea water (Panchal, 1988) and is said to prevent corrosion and biofouling.

In order to delay the initial adhesion, monomolecular coatings may be obtained by wetting the surface with a suitable liquid that decreases the surface free energy before starting the heat exchanger operation. This is seldom satisfactory, since fluid flow rapidly removes the protecting monolayer. Corrosion inhibitors are useful in preventing the formation of oxide layers that promote cell attachment.

Velocity is a key factor in the design and operation of heat exchangers. Moderate to high velocities should be used to reduce biofouling : recommended values range from 1.5 to 3 m/s.

The problem mentioned in the last paragraph of section 3.5. can be overcome by recycling part of the water flow in the tubes during the initial period of operation, in order to maintain adequate fluid velocities. Also, "temperature excursions" (sudden changes in temperature) can be promoted in order to disturb microbial activity and to cause the detachment of the biolayer from the surface.

The spacing between baffles in a shell and tube heat exchanger is important in mittigating shell-side deposition (Figure 3), by eliminating the low velocity regions, the so-called "dead zones" (Chenoweth, 1988).

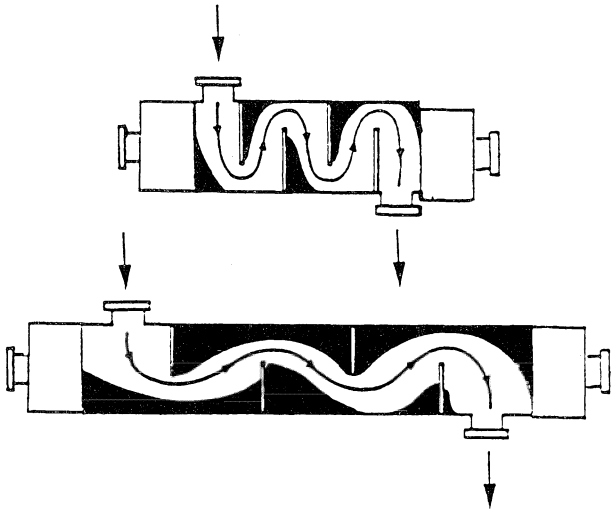


Figure 3 - Effect of the spacing between baffles in the shell side of a heat exchanger

In general, the fluid most prone to cause fouling should circulate within the tubes, since shear stresses are usually higher, it is easier to clean the inside of the tubes and "dead zones" are not frequent here.

On-line mechanical cleaning --- such as the periodic circulation of sponge rubber balls ("Tapprogge System") or plastic brushes through the tubes --- avoids the build up of biofilms and is a practical method used in some types of heat exchangers (e.g., in large condensers).

4. Final Remarks

It is not always feasible to eliminate biofouling in industrial situations, even when science has the methods to achieve this purpose. In fact, economic or environmental considerations may rule over technical possibilities or scientific advancements. Substantial reductions in biofilm build up may, however, be obtained if the appropriate design and operating conditions are met. In many cases, one should probably start by implementing techniques to monitor biofilm development in the equipment in order to gather the necessary information for biofouling control.

Unpredicted shutdowns arer often extremely expensive. Thus, it is quite important to be able to predict the rate of biofilm formation so that an appropriate cleaning schedule or an adequate biocide dosing can be effectively and economically applied. A deeper knowledge of biofouling mechanisms as well as the development of practical mathematical models should be sought in order to improve the prevention and control of this unwanted phenomena.

Nomenclature

A - heat transfer area, m^2
F - mean temperature correction factor
h_h, h_c - convective heat transfer coefficients for the hot and cold fluids, respectively, W/m^2K
k_w - wall thermal conductivity, W/mK
q - heat transfer rate, W
R_f - thermal resistance of the biofilm, m^2K/W
T_h, T_c - temperatures of the hot and cold fluid, respectively, K
U_0 - overall heat transfer coefficient with a clean wall, m^2K/W
U - overall heat transfer coefficient with a fouled wall, m^2K/W
x_w - thickness of the wall, m

References

Baier, R.F. (1979) 'Modification of surfaces to reduce fouling and/or improve cleaning', in Bitton and Marshall (eds.), Adsorption of Microorganisms to Surfaces, Wiley, New York.

Battaglia, P.J., Bour, D.P. and Burd, R.M., 'Biofouling control practice and assessment', Proj. 1132-1, Eletric Power Research Institute, Palo Alto, California.

Bott, T.R. (1992) 'The use of biocides in industry', in Melo *et al.* (eds.), Biofilms-Science and Technology, Kluwer Academic Publishers, Dordrecht, The Netherlands, this volume.

Bott, T.R. and Melo, L.F. (1992) 'Particle-bacteria interactions in biofilms', in Melo *et al.* (eds.), Biofilms-Science and Technology, Kluwer Academic Publishers, Dordrecht, The Netherlands, this volume.

Bott, T.R. and Pinheiro,M.M. (1977) 'Biological fouling-velocity and temperature effects', Canadian J. Chem. Engin., 55, 473.

Bush, A.W. (1971) 'Aerobic biological treatment of waste waters',Gulf Publishing Co., Houston, Texas.

Chamberlain, A.H. (1992) 'Biofilms and Corrosion', in Melo *et al.*(eds.), Biofilms-Science and Technology, Kluwer Academic Publishers, Dordrecht, The Netherlands, this volume.

Characklis, W.G. (1980) 'Biofilm development and destruction', Final Report EPRI Cs-1554, Project RP 902-1, Electric Power Research Institute, Palo Alto, California.

Characklis, W.G. (1990) 'Biofilm Processes', in Characklis and Marshall (eds.), Biofilms, Wiley, pp. 195-222.

Characklis, W.G. Nimmons, M.J. and Picologlu, B.F. (1988), Journal of Heat Transfer Engineering, 3, 23.

Chenoweth, J.M. (1988) 'General design of heat exchangers for fouling conditions', in Melo, Bott and Bernardo (eds.) Fouling Science and Technology, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 477-494.

Dexter, S.C. (1979) 'Influence of substratum critical surface tension on bacteria adhesion - in situ studies', J. Colloid and Interface Science, 70, 2.

Duddridge, S.C., Kent, C.A. and Laws, J.F. (1981) 'Bacterial adhesion to metallic surfaces', in Proceedings of the conference on Progress in the Prevention of Fouling in Industrial Plant, University of Nottingham, pp. 137-153.

Duddridge, S.C., Kent, C.A. and Laws, J.F. (1982) 'Effects of shear stress on the attachment of *Pseudomonas fluorescens* to stainless steel under defined flow conditions', Biotech. & Bioengin., 24, 153.

Eaton, A., Chamberlain, C. and Cooney, M. (1980) 'Colonization of Cu-Ni surfaces by microfouling organisms', in J.F. Garey et al. (eds.), Condenser Biofouling Control, Ann Arbour Science.

Garey, J.F. (1979) 'Biofouling control investigations', Interim Report EA 1082,Research Project 1372-1, Electric Power Research Institute.

Hatori, R. and Hatori, T. (1981) 'Effect of a liquid-solid inter face on the life of microorganisms', Ecological Review, 16 (1), 63.

Howell, J.A. and Atkinson, B. (1976), Water Research, 10, 307.

Lowe, M., Duddridge, J.E., Pritchard, A.M. and Bott, T.R. (1984) 'Biological - particulate fouling interactions : effects of suspended particles on biofilm development', in Proceed. of the 1st UK National Heat Transfer Confer., Leeds, pp. 391-400.

Lund, D. and Sandu, C (1981) 'State of the art of fouling : heat transfer surfaces', in Hallström, Lund and Trägardh (eds.), Fundamentals and Applications of Surface Phenomena Associated with Fouling and Cleaning in Food Processing, Lund University, Lund, Sweden, pp. 27-56.

McCaughey, M.S., Thau, A., Characklis, W.G. and Goodman, D.(1987) 'An evaluation of condenser tube fouling at an estuarine nuclear power plant', Joint Power Generation Conference, ASME, Miami, Florida.

Pinheiro, M.M., Melo, L.F., Bott,T.R., Pinheiro, J.D. and Leitäo, L. (1988) 'Surface phenomena and hydrodynamic effects on the deposition of *Pseudomonas fluorescens*, Can. J. Chem. Eng., 66, 63-67.

Oliveira, R., Melo, L., Pinheiro, M. and Vieira, M.J. (1992) 'Surface interactions and deposit growth in fouling of heat exchangers', Corrosion Reviews, in press.

Panchal, C.B. (1988) 'Experimental investigation of marine biofouling and corrosion for tropical seawater', in Melo, Bott and Bernardo (eds.) Fouling Science and Technology, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 241-247.

Sanders, W.M. (1964), Ph.D. Thesis, John Hopkins Univ.,Baltimore,USA.

Stathopoulos, N. (1981) 'Influence of temperature on biofilm processes', M.Sc. Thesis, Rice University, Houston, Texas,USA.

Thackery, P.A. (1980) 'The cost of fouling in heat exchange plant', Effluent and Water Treatment J., 20(3).

Trulear, M.G. and Characklis, W.G. (1982), J. Water Poll. Control Federation, 54, 1288.

Vieira, M.J., Oliveira, R., Melo, L., Pinheiro, M. and van der Mei, H.(1992a) 'Adhesion of *Pseudomonas fluorescens* to metallic surfaces', J. Dispersion Science and Technology, 13(4), 437-445..

Vieira, M.J., Oliveira, R., Melo, L.F., Pinheiro, M.M. and Martins, V. (1992b) 'Adhesion of biofilms formed by *Pseudomonas fluorescens*', submitted to Colloids and Surfaces.

Vieira, M.J., Melo, L.F. and Pinheiro, M.M. (1992c) 'Biofilm formation - hydrodynamic effects on internal diffusion and structure', submitted to Biofouling.

PROPERTIES AND APPLICATIONS OF *THIOBACILLUS FERROOXIDANS* BIOFILMS

L. N. NIKOLOV
University of Sofia
Biological Faculty
8 Dr. Tsankov Str.
Sofia 1421, Bulgaria

1. Introduction

The bacterial oxidation of ferrous iron using chemolithotrophic *Thiobacillus ferrooxidans* generally expressed by the equation:

$$Fe^{2+} + 0.5O_2 + 2H^+ \xrightarrow{\text{Thiob.ferroox.}} Fe^{3+} + H_2O \qquad (1)$$

was initially assumed as the first stage of the indirect mechanism of metal leaching of low grade sulfide ores (Olson and Kelly, 1986). Recently the reaction (1) is drawing the bioprocess engineers interest in the diversity of applications. The generation of ferric ions solutions from $FeSO_4$ is turned out to be the core of quite different industrial applications starting with acid mine drainage waste water treatment (Olem and Unz, 1977; Nakamura, 1986; Muriyama, 1987) and hydrometallurgy of uranium (Olson and Kelly, 1986) and ending with H_2S tail gases purification (Imaizumi, 1986; van Lookeren, 1986) or with the ferric ions containing coagulant production.

These applications are economically justified due to the ability of the *Thiobacillus ferrooxidans* to form biofilms and in such a way to ensure a basis for the bioprocess engineers to develop biofilm reactors with technological parameters acceptable from practical point of view. The engineering and technological gist of above-mentioned industrial implementations are very close to these of municipal waste water treatment. However the properties of the biofilms formed by *Thiobacillus ferrooxidans* are rather different in comparison with the other biofilms well-known in science and practice of spontaneously fixed biomass.

2. Aim

The aim of this work is to reveal the most interesting *Th. ferrooxidans* biofilms properties from engineering point of view and to show their application on high performance biofilm reactor design and exploitation on the basis of our own experience.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 511–519.

3. Materials and methods

3.1 BACTERIA, MEDIA AND SUPPORTS

Two bacterial strains of *Thiobacillus ferrooxidans* have been used: No 737 of Bulgarian National Collection of Microorganisms and Cell Cultures (Nikolov, 1988a) and an industrial strain (Nikolov, 1986a) kindly submitted by Bulgarian Association of Metallurgy.
Both 9K Silverman and Lundgren (Valkova et al., 1982; Nikolov et al., 1986b; Nikolov et al., 1988b; Nikolov et al., 1989a) or Tuovinen and Kelly media (Nikolov et al., 1986a; Nikolov and Karamanev, 1990a; Nikolov and Karamanev, 1990b; Nikolov et al.,1990) have been used in most of kinetic experiments in laboratory scale. In the performance of industrial scale experiments only the Tuovinen and Kelly meduim has been used.
Three types of inert supports have been implemented: a) PVC as a material for biodisks (Valkova et al., 1982; Nikolov et al., 1986b; Nikolov et al., 1986c) and turnings for packed bed bioreactors (Nikolov et al., 1988a); b) Expanded polystyrene spheres with density 50-800 kg/m^3 for inverse fluidized bed bioreactors (Nikolov and Karamanev, 1987; Karamanev and Nikolov, 1988; Nikolov and Karamanev, 1990a; Nikolov and Karamanev, 1990b) and nylon spheres with density 950 kg/m^3 for upflow fluidized bed bioreactor (Nikolov et al., 1986d); c) Stainless steel turnings for packed bed bioreactors (Nikolov et al., 1986a).

3.2. BIOREACTORS

Six different types of bioreactors have been studied: a) Biodisk reactor (fig.1a, a=100 m^2/m^3) (Valkova et al., 1982; Nikolov et al., 1986c) and its new modification (fig.1b, a=120-130 m^2/m^3) (Nikolov et al., 1986b); b) Airlift draft tube bioreactors with package bed in the annulus (fig.1c, a=400 m^2/m^3) (Nikolov et al., 1986a) and with inverse fluidized bed of light polystyrene spheres - IFBBR (fig.1d, a=1700-2000 m^2/m^3) (Nikolov and Karamanev, 1987); c) Upflow fluidized bed (fig.1e, a=850 m^2/m^3) (Nikolov et al., 1986d) and packed bed with PVC turnings (fig.1f, a=450 m^2/m^3) (Nikolov et al., 1988a) bubble column.

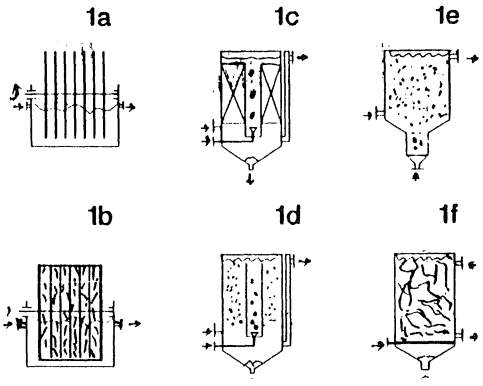


Figure 1. Shemes of different bioreactor designs

3.3 BIOFILM PROPERTIES MEASUREMENTS AND OBSERVATIONS

3.3.1 *Physical properties*. The biofilm thickness has been measured directly by means of micrometer, microscope (Nikolov et al., 1986c; Nikolov and Karamanev, 1987; Nikolov et al., 1988a) or have been assessed on the basis of 20 measurements of the biomass volume (Karamanev and Nikolov., 1988). Average biofilm density has been determined by means of pycnometer (Nikolov et al., 1989a). The biofilm solid structure properties have been determined after drying at 110°C to the constant weight using "Micrometrics Auto-pore 9200 V2.03" device (USA). The *Th. ferrooxidans* biofilm wearlessness has been measured implementing the techniques of turning drum (Paranskii et al. 1969).

3.3.2 *Physico-chemical properties*. The solubility of the biofilms formed in biodisk reactors in different concentrations of Fe^{2+} ions (4, 9 and 14 kg/m^3) has been studied under static conditions and on the shaker (G 10 "New Brunswick" (USA)) at 3.33 sec^{-1} in concentrated mineral acids (HCl, H_2SO_4, HNO_3) and their combinations. The determination of Fe^{2+} and Fe^{3+} ions has been carried out by colorimetric analysis (Nikolov and Karamanev, 1987) or by the bichromate method (Nikolov et al., 1988a). The reaction (1) velocity for continuous regime has been calculated by equation:

$$r_v = (S_{in} - S_{out}).D \qquad (2)$$

3.3.3 *Microbial properties*. Bacteria viable count has been determined by 3 tubes MNP technique in 9K medium (Nikolov et al., 1988a; Vulkova-Vulchanova et al., 1989). The number of *Th. ferrooxidans* in biofilm solids has been assessed by the method described in details in the study of the packed bed bioreactor (Nikolov et al., 1988a). Aerobic heterothroph microflora accompanying *Th. ferroxidans* and its influence has been studied by procedures discussed in another work (Vulkova-Vulchanova et al., 1989). The bacterial activity has been estimated on the basis of the kinetics data.

4. Results and discussion.

4.1 *Biofilm properties as a basis for high performance bioreactor design and developments*. The study on the mechanical properties using the turning drum techniques (Paranskii et al., 1969) have proved that the biofilms of *Th. ferrooxidans* posses wearlessness (relative loss of weight of 3.8%) - typical for many chemical solid catalysts and adsorbents. This property has been used to develop a new modification of the biodisk reactors (fig.1b) (Nikolov et al., 1986b) with effective reuse of the biofilms. The new bioreactor has allowed the sloughed biofilm fragments to stay long time in the aeration zone of the biofilm reactor rotating with the biodisks. It can be seen on the fig.2 that the productivity of the modified biodisk reactor is more stable and higher than that of the classical one. However the experimental data of another study has shown that the biodisk reactors design could be applied when the input Fe^{2+} concentrations is not higher than 14 kg/m^3 (Nikolov et al., 1986c).
Long time work (more than 5 months) with laboratory scale biodisk reactors allowed to obtain rather deep layers of sediments on their bottoms formed by sloughed biofilms and the biofilm on the side walls of the submerged zone of the apparatus. This has decreased the working

volume of the bioreactor. The similar effect was reported elsewhere (Olem and Unz, 1977). This has provoked the study on the solubility of the biofilms in different mineral acids and 10% of oxalic acid in water.

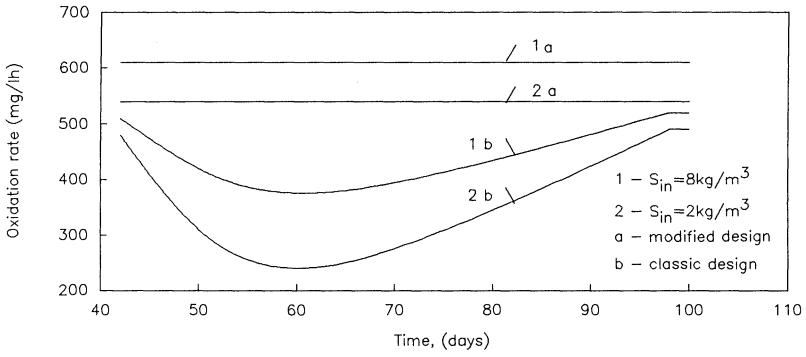


Figure 2. Comparison of the effectiveness of the biodisk reactors

The experimental results have shown that 2 hours were enough for full purge of the surplus biofilm quantity even under static conditions using mixtures of HCl and HNO_3. For H_2SO_4 and HCl mixtures and for oxalic acid water solutions this time could be 1-2 days. Thus it has been found a method for periodical purge of the surplus biomass and sediments (Nikolov et al., 1987).

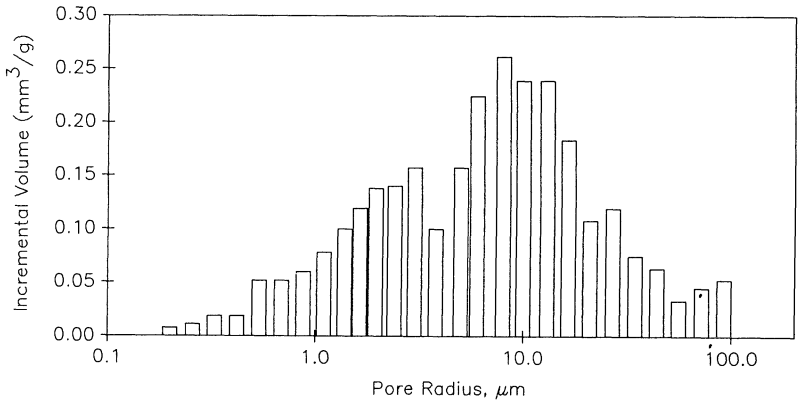


Figure 3. Distribution of pores volumes in the biofilms

An inspection of biofilm fragments by microscope has shown that its density has decreased to the outermost layers and there is quite possible existence of certain porous structure. The results of porometry measurements have confirmed the availability of internal surface (up to $1.4.10^4$ m^2/kg) and distribution of pore volumes shown on fig.3. The parameters of the porous structure have not been found to depend on the conditions under which the biofilm has been formed (Nikolov et al., 1989a).
In the experiments with the new modification of the biodisk reactor it has been assumed that this design ensure appropriate conditions for the most active microorganisms adsorbed at the outermost layers of sloughed biofilm fragments to take part in the oxidation process. Later this assumption has been confirmed by the results of an investigation on the activity of biofilms of *Th. ferroxidans* during their formation (Nikolov et al., 1988a). It is visible from fig.4 that after the washing out of the microorganisms from the outermost layers of the biofilm on the 14th day the ferrous iron oxidation velocity is decreasing (line 1 and 2).

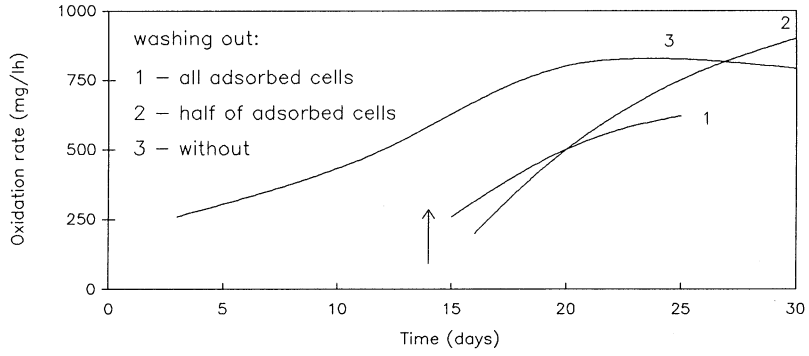


Figure 4. Influence of adsorbed cells on the dynamics of biofilm formation

This result has explained also the higher productivity of the airlift bioreactors (fig. 1c and 1d) where was found existence of suitable situation for spontaneous fixation and for saving the outermost layers of microorganisms intact due to low shear stress. On the contrary in upflow fluidized bed bioreactor (fig.1e) (Nikolov et al., 1986c) the shear stress has been considered to be much more higher. Moreover the design of the IFBBR (fig.1d) has provided very good conditions for biofilm thickness control due to three phase upflow fluidized bed in the draft tube (Nikolov and Karamanev, 1990a; Nikolov and Karamanev, 1990b). Using this bioreactor it was found that working without any external or internal diffusion limitations in the biofilm of *Th.Ferrooxidans* the velocity of the reaction (1) could be higher up to 10 times in comparison with suspended culture up to 12 kg/m^3 for Fe^{2+} concentrations.
At the same time it has been discovered that the kinetics has not been sensitive to the change of the temperature (obtained almost simultaneously with Nakamura at al. (Nikolov and Karamanev, 1987)), pH and ferrous and ferric ions concentrations (Karamanev and Nikolov,

1988). These effects have turned out to be very useful especially when it concerned to development of so called robust industrial processes like these of biohydrometallurgy, mine drainage waters treatment where the major part of the technology lines is in the open.
The intensification of metal leaching has imposed the necessity to produce ferric ions solutions in the form $Fe_2(SO_4)_3$ with concentrations as high as possible. A successful decision of this task has been found by the use of both airlift packed bed laboratory scale biofilm reactors and IFBBR (fig.1c and 1d). However it has been found out by computer simulations using a mathematical model of the IFBBR (Chavarie et al., 1986) that along with the above described advantages the implementation of the airlift principle in biofilm reactor design could create some problems like the lack of oxygen in the lower annulus bed layers when the apparatus is rather high or the concentrations of ferrous and ferric ions are over 20 kg/m^3. This has been the main reason to look for another modification of biofilm reactor. It has been supposed by analogy with the airlift bioreactors with stainless steel turnings (fig. 1c) that in bubble columns with beds of turnings (fig.1f) low shear stress zones could exist in microscale and a biofilm could be formed in spite of the intensive hydrodynamic of gas-liquid-solid systems of this kind. The bubble column design has been attractive with the high oxygen mass transfer characteristics. The realization of these considerations has given a very simple and effective biofilm reactor - a bubble column with packed bed of PVC turnings as support. This bioreactor has been studied in laboratory scale (Nikolov et al., 1988a; Nikolov et al., 1988b; Nikolov et al., 1989b; Nikolov et al., 1990) ($1.16.10^{-3}$ m^3 working volume) and under industrial conditions (18 m^3 working volume). Working under unsterile conditions and imitating in such a way in the laboratory the real industrial situation an existence of a certain microflora in this bioreactor was found. Anyhow it has been proved that when the concentrations of these accompanying microorganisms was low (which is typical for the operating conditions in biohydrometallurgy) they could not defer the oxidation process (Nikolov et al., 1988b; Vulkova-Vulchanova et al., 1989). All this explain the very good characteristics of bubble column working under laboratory and industrial conditions with input ferrous iron concentrations even in the range 50-70 kg/m^3. But uncontrolable biofilm growth on PVC turnings in the packed bed has imposed the necessity to implement the above-mentioned method of appropriate purge of the surplus biofilm (Nikolov et al., 1987).
The study of the relation between the biofilm properties and the bioreactor design has given a set of bioreactors ensuring the highest bacterial oxidation velocity of 1.5 to 6.0 kg/m^3 published in the literature. These results could be successfully applied in the development of new high efficiency industrial methods in different fields of the practice.

4.2 *The biofilm of Thiobacillus ferrooxidans influences the ability of its own microorganisms.*
The experiments in the field of bioreactor design for bacterial oxidation of high concentrated ferrous ions solutions have shown considerable difference in the behavior of suspended and spontaneously fixed *Thiobacilus ferrooxidans*. It has been discovered that suspended bacteria have not been able to oxidize ferrous ions in solutions with concentrations higher than 30 kg/m^3 (Nikolov et al., 1986a; Nikolov et al., 1988a;) while if the same bacteria have been in biofilm they could be trained to work with concentrations up to 70 kg/m^3. This fundamental result has allowed to realize for the first time in the practice a bacterial ferrous ions oxidation of such a high concentration (Nikolov et al., 1986a; Nikolov et al., 1988a; Nikolov et al., 1988b; Nikolov et al., 1989b; Nikolov et al., 1990). The study of the oxidation process

kinetics in cascade of bubble column bioreactors (Nikolov et al., 1990) in the very large span of Fe^{2+} concentrations (0-41 kg/m^3) after the model discrimination has given as most statistically reliable the following equation:

$$r_v = \mu_{max} X/Y.S/(K_s + S + S^2/K_{si}) \quad (3)$$

The results of another study on kinetics of resuspended cells have revealed that the *Thobacillus ferrooxidans* once fixed in a biofilm and had oxidized high concentrations of ferrous ions have kept their ability to live and work in suspension under such extreme conditions in a year (Nikolov and Karamanev, 1990c; Nikolov and Karamanev, 1992). The most appropriate kinetic equation describing the experimental data has been proved to be:

$$D = \mu_{max}/(S + I + S^2/K_{si}) \quad (4)$$

It could be supposed that in contrast to the fixed state of the bacteria the reaction velocity after resuspension is influenced by the product.

4.3 *Mathematical modelling of the biofilms of Thiobacillus ferrooxidans.* The biofilms have been modelled using obtained properties. The existence of the porous structure has been the main reason to consider an effective diffusivity coefficient equal to the half of the diffusivity coefficient of the oxygen or ferrous ions in water. This hypothesis has been the basis of the mathematical models of IFBBR (Chavarie et al., 1986; Nikolov and Karamanev, 1990a) and of the biodisk reactor (Slavchev et al., 1987). The computer simulation have given sufficient coincidence with the laboratory experimental results. Moreover using the idea of the porous structure it has been suggested that bacteria attach by physical adsorption on the pores surface of the inert solid mineral framework (Karamanev, 1991). The mathematical model has explained the interactions between adsorbed microorganisms and those which have been in suspension in equilibrium with them. In another study (Karamanev and Nikolov, 1991) it has been proved that the capability of *Th. ferrooxidans* to oxidize ferrous iron at low concentrations in biofilm and in suspension was the same. The difference in the oxidation rates in high concentrations could be explained by the difference of the concentrations of the substrates and of the biomass. However the change of ability of the *Thiobacillus ferrooxidans* cells which has already been in biofilms to oxidize very high concentrations of ferrous ions is still waiting to be understood.

5. Nomenclature

D - dilution rate;
I - ferric ions concentration;
K_s - saturation constant;
K_{si} - constant of substrate inhibition;
μ_{max} - maximal specific growth rate;
r_v - reaction velocity;
S - ferrous ions concentration;
X - biomass concentration;
Y - yield coefficient

6. References

Chavarie, C., Karamanev, D., Nikolov, L., Champagne, J. (1986) "Simulation d'un Reacteur a Biofilm a Fluidization Inversee" (French) 4emes Journees Europeennes sur la Fluidization, Toulouse, France, 254-265.

Imaizumi, T. (1986) "Some Industrial Applications of Inorganic Microbial Oxidation in Japan." Biotechnol. Bioeng. Symp., **16**, 367-371.

Karamanev, D., Nikolov, L. (1988) "Influence of Some Physicochemical Parameters on Bacterial Activity of Biofilm. Ferrous Iron Oxidation by *Thiobacillus ferrooxidans*", Biotechnology and Bioengeneering, **31**, 4, 295-300.

Karamanev, D. (1991) "Model of the Biofilm Structure of *Thiobacilus ferrooxidans*" J. Biotechnol., **20**, 51-64.

Karamanev, D., Nikolov, L. (1991) "A Comparison Between The Reaction Rates in Biofilm Reactors and Free Suspended Cells Bioreactors", Bioprocess Eng., **6**, 127-130.

Muriyama, T., Konno, Y., Sakata, T., Imazuni, T. (1987) "Application of Immobilized *Thiobacillus ferrooxidans* for Large-Scale Treatment of Acid Mine Drainage." Methods Enzymol., **136**, 530-540.

Nakamura, K., Noike, T., Metsumoto, J. (1986) "Effect of Operation Conditions on Biological Fe^{2+} Oxidation by *Thiobacillus ferrooxidans.*" Water Res., **20**, 73-77.

Nikolov, L., Penev, T., Popova, V., Ivanov, A., Kuzmanov, L. (1986a) "Microbial Oxidation of Ferrous Ions in a Spontaneously Fixed Biomass Bioreactor." Biotech. and Bioindustry (Bulg.), **2**, 14-16.

Nikolov, L., Valkova, M., Penev, Ts., Kuzmanov, L. (1986b) "A Possibility of Improvement of the Design of a Biodisk Reactor." Biotech. and Bioindustry (Bulg.), **3**, 28-31.

Nikolov, L., Mehochev, D., Dimitrov, D. (1986c) " Continuous Ferrous Iron Oxidation by Thiobacilus ferrooxidans in Rotating Biological Contactors" Biotechnol. Letters, **8**, No 10, 707-710.

Nikolov, L., Mehochev, D., Kuzmanov, L., Karamanev, D., Todorov, T. (1986d) Bulg. Inv. No 73209 / 23 .1.1986.

Nikolov, L., Karamanev, D. (1987) "Experimental Study of the Inverse Fluidized Bed Bioreactor". The Canadian Journal of Chemical Engineering, **65**, 214-217.

Nikolov, L., Kolev, S. (1987) "Investigation of the Type of Flow Pattern in a Rotating Disk Reactor", Chemical and Biochemical Engeneering Q., **1**, 1, 39-41.

Nikolov, L., Proevska, E., Mehochev, D., Dimitrov, D. (1987), "Studies on the Solubility in Mineral Acids of Sediments of Spontaneously Fixing Microorganisms from *Th. ferrooxidans Species",* Biotech. and Bioindustry (Bulg.), **6**, 42-46.

Nikolov, L., Valkova-Valchanova, M., Mehochev, D. (1988a) "Oxidation of High Ferrous Iron Concentrations by Chemolithotropic *Thiobacillus ferrooxidans* in Packed Bed Bioreactors", Journal of Biotechnology, **7**, 87-94.

Nikolov, L., Valkova-Valchanova, M., Mehochev D., Todorov, T. (1988b) "Production of High Concentrations of Ferric Sulphate by Chemolithotropic *Thiobacillus ferrooxidans*", 8 Int. Biotechnology Symposium, Paris, , Abstract Book, No 12, p.7.

Nikolov, L., Mehochev, D., Dimitrov, D., Proevska, E. (1989a) "Chemolithotrophic *Thiobacillus ferrooxidans* Forms Unusual Biofilms", Int. Symp.: Physiology of immobilized cells, Wageningen, The Netherlands, Dec.10-13, , p.45.

Nikolov, L., Valkova-Valchanova, M., Mehochev, D., Penev, T. (1989b) "Continuous Oxidation of High Ferrous Iron Concentrations by Spontaneously Immobilized *Thiobacillus ferrooxidans*", 5 Conf. of Chemistry and Biotechnol. of Biologically Active Natural Products, Varna, Sept.18-23, , 455-459.

Nikolov L., Karamanev, D., Valkova-Valkanova, M. (1990) "Kinetics of the Oxidation of High-Concentrated Ferrous Ions by *Thiobacillus ferrooxidans*", 5 European Congress on Biotechnology, Copenhagen, Denmark, , p. 391.

Nikolov L., Karamanev, D. (1990a) "The Inverse Fluidized Bed Biofilm Reactor : A New Laboratory Scale Apparatus for Biofilm Investigations", Journal of Fermentation and Bioengineering (Japan), **69**, 265-269.

Nikolov L., Karamanev, D. (1990b) "The Inverse Fluidized Bed Biofilm Reactor: A New Tool for Biofilm Investigations", Physiology of Immobilized Cells (J.A.M. DeBont, ed.), Elsevier, The Netherlands, 461-465.

Nikolov L., Karamanev, D. (1990c) "Change of Microbial Activity After Immobilization of Microorganisms", Physiology of Immobilized Cells (J.A.M. DeBont, ed.), Elsevier, The Netherlands, 649-660.

Nikolov, L., Karamanev, D. (1992) "Kinetics of the Ferrous Iron Oxidation by Resuspended Cells of *Thiobacillus ferrooxidans*", Biotechnol. Progress (in press).

Olem, H., Unz, R.F. (1977) "Acid Mine Drainage Treatment with Rotating Biological Contactors." Biotechnol.Bioeng., **19**, 1475-1491.

Olson, J.G., Kelly, R.M. (1986) "Microbiological Metal Transformations: Biotechnological Applications And Potential." Biotechnol. Prog., **2**, 1-9.

Paranskii, S., A., Veden'eva, A., I., Bessonsov, A. (1969) "On the Solidity Assessment of the Catalysts under the Conditions of Wear Influence Forces" Kinetika i Katalis " (Russ.), **10**, No 1, 175-182.

Slavchev, S., Miladinova, S., Nikolov, L. (1987) "Modeling of Biodisk Reactors", Biomechanics (Bulg), **24**, 78-82.

Valkova, M., Nikolov, L., Todorov, Tz., (1982) " Microbial Ferrous Ions Oxidation in Rotating Bioreactor." Annulair de L'Universite de Sofia "Kl. Ohridski"(Bulg.), **75**, 58-65.

van Lookeren, C., Constant, J. (1986) "Gas Treatment Process." European Pat.Appl. No 86201844.7

Vulkova-Vulchanova, M., Nikolov, L., Penev, T. (1989) "Aerobic Heterothrophs in Bioreactor with Spontaneously Fixed Biomass of *Th.. ferrooxidans*" Cmt.Rend.Bulg.Acad.Sci., **40**, No 12, 69-72.

BIOFOULING IN STIRRED TANK REACTORS - EFFECT OF SURFACE FINISH

D.C. REID and T.R. BOTT
School of Chemical Engineering
University of Birmingham
Edgbaston
Birmingham B15 2TT. UK

R. MILLAR
Biochemical Engineering Section
NEL Executive Agency
DTI
East Kilbride
Glasgow G75 OQU. UK

1. Introduction

During 1990 ninety bioprocess plant manufacturers and users were asked to comment on problems experienced with biofouling (Reid et al 1991). A 35% response rate was achieved. Of this return 19% indicated having experienced biofouling problems; 27% had no such problems while 59% were unaware of experiencing biofouling or related problems. The majority of plant in use was constructed from stainless steels. Surface finishes for plant ranged from Ra (0.1) - mirror finish to Ra (6.3) - Vaccublast/unfinished.

A number of authors have indicated that biofouling was an important variable requiring quantification from a theoretical standpoint (Reid et al 1991). Another aspect of the phenomenon was the economic one - as a reduction in surface finish would reduce the production costs of bioprocess equipment (Timperley 1984). The present study was undertaken to examine the role of surface finish on the biofouling phenomenon.

2. Materials and Methods

The influence of surface finish was investigated using a Stirred Tank Reactor (STR) system (Chemap Alfa Laval). Stainless steel tanks were constructed with internal surface finishes of Ra (3.8) rough; Ra (2.3) intermediate; and Ra (0.06) smooth, where Ra (μm) is the Arithmetic Mean Deviation of the Profile (British Standard 1134) average roughness height, with sample length of 0.8 mm. Before each fermentation the tanks (shells) and other components were cleaned with acetone and rinsed under cold running water for five minutes. Following cleaning the fermenter was assembled, medium added and the unit sterilised at 115°

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 521–526.

for 20 minutes. Once the medium cooled to the process temperature (30°C) the fermenter was inoculated with 100 ml of a 72 hour starter culture previously grown in a 250 ml shake flask.

Test cultures used throughout were *Bacillus subtilis* (AJ1992) grown on Neish medium (Neish et al 1945); *Saccharomyces cerevisiae* (K2ND) grown on commercial wort (Brewing Products UK Ltd) (Arcay-Ledezma and Slaughter 1984); *Pseudomonas fluorescens* (NCIB9046) grown on media described by Mott and Bott (1990); and *Penicillium chrysogenum* (M9) grown on Hockenhull medium (Hockenhull 1959).

Bacillus, *Pseudomonas*, and *Penicillium* fermentations were maintained at 30°C and mixed at 350 r/min using a Rushton-type agitator. The dissolved oxygen, pH, temperature and optical density were monitored. At the end of each run the vessel was drained and the shell removed for sampling. *Saccharomyces* fermentations were carried out in static tanks for 7 days (to mimic the industrial situation).

Biofouling was assessed using the modified method of Maw and Smith (1986). Predefined areas (314 mm^2) inside the shells were scoured with melamine foam. The foam was then assayed for protein content using the method of Lowry et al (1951). The sampling pattern is shown in Fig. 1. This pattern was modified for sampling the *Saccharomyces* fermentation (the top and above bulk locations were not sampled).

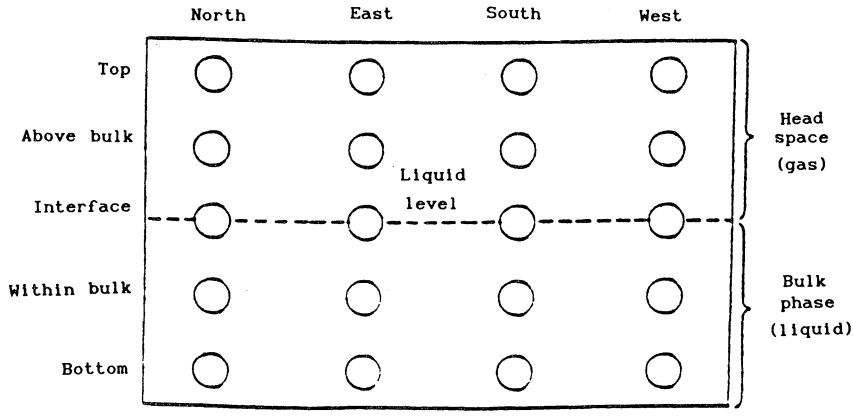


Figure 1. Sample positions

3. Results

The mean values from duplicate runs with the test organisms were analysed statistically by Analysis of Variance (ANOVA) using transformed

(log (e)) data. The results from this analysis are given in Tables 1 and 2. Confidence intervals of 95 per cent were used as the statistical cut-off in the following discussion (ie. any p-values less than 0.05 were significant).

TABLE 1. p-values from ANOVA model

	Organism			
Source	*Bacillus subtilis*	*Penicillium chrysogenum*	*Pseudomonas fluorescens*	*Saccharomyces cervisiae*
Surface	0.001	0.024	0.001	0.173
Vertical	0.000	0.000	0.000	0.001
Horizontal	0.308	0.585	0.585	0.901

TABLE 2. Mean protein values from ANOVA model

	Mean protein value (μg BSA)			
	Bacillus subtilis	*Penicillium chrysogenum*	*Pseudomonas fluorescens*	*Saccharomyces cervisiae*
Surface				
Ra (0.06)	240	209	220	701
Ra (2.37)	305	256	151	654
Ra (3.8)	356	299	217	519
Vertical position				
Top	379	248	430	-
Above bulk	407	513	376	-
Interface	277	517	200	920
Within bulk	222	132	86	516
Bottom	241	117	97	502
Horizontal position				
North	336	283	212	665
East	274	252	180	635
South	296	244	196	588
West	284	232	187	593

4. Discussion

4.1 *BACILLUS SUBTILIS*

The amount of adsorbed protein appears to increase with increasing surface roughness. The p-value from the ANOVA (Table 1) indicates that the surface effect has a highly significant influence on variation, indeed the p-value suggests that this effect has a probability of greater than 99.9 per cent of being caused by a factor other than chance. The components of the variation observed may be identified by examining Table 2. This showed an increase in adsorbed protein with increasing roughness.

Results presented in Table 1 also indicate that the vertical distribution was highly significant in producing the observed variation with a marked increase in the top and above bulk samples (Table 2). The horizontal component did not appear to exert any significant influence.

4.2 *PENICILLIUM CHRYSOGUENUM*

Adsorbed protein values show a dramatic increase in the amount of absorbed protein from the interface region upwards. Results indicate that the peak protein values appear in the Above Bulk sample. Unlike *Bacillus subtilis* there was no clearly differentiated pattern between the surfaces. Table 1 indicates that both the surface and vertical samples have a significant effect on the variation observed. The mean values (Table 2) show clearly that the significant variation in surface effects was due to Ra (0.06). Also there were three distinct levels of adsorbed protein for the vertical sample corresponding to the bulk phase area; the interface; and the headspace area.

These patterns can be interpreted by considering the 'morphology' of the fermentation. The *Penicillium*, while growing in the bulk phase, formed a mycelial mat over the interface, this mat formed over time thus during the initial growth period splashing would have been able to transport material to all regions of the headspace areas. Once the mycelial mat was established, this mechanism was unable to continue. The already established biomass in the headspace continued to grow merging with the mycelial mat at the 'interface' and 'above bulk' sample regions thereby causing the pattern of adsorbed protein found.

4.3 *PSEUDOMONAS FLUORESCENS*

Results show the familiar increase in adsorbed protein in the headspace region of the various shells. From this figure the increases recorded did not appear as great for Ra (2.3). This occurred when one of the fermentations did not produce as much foaming as in the other examples. In order to try and improve this situation another run was made with the Ra (2.3) shell. This run did produce a foam and these data were used with the original 'foaming' run to produce the statistics discussed.

Table 1 indicates that the surface and vertical position were both highly significant. Table 2 shows that for the surface effects, despite using 'foaming data', Ra (2.3) caused the significant variation due to the low overall mean value found. Vertical effects increased along the

shell from bottom to top with the horizontal effects having no significant influence on the variation encountered.

4.4 *SACCHAROMYCES CEREVISIAE*

The fermentations for *Saccharomyces* were carried out for seven days in static tanks. This meant that there were no samples taken in the above bulk and top sample regions as no mechanisms existed to transport material to such sites. The sample matrix was thus 3 by 4 (as opposed to 5 by 4 in all cases above).

The results showed a marked increase in the amount of adsorbed protein for shell Ra (0.06) compared to the other two shells. The data indicate that while in the bulk phase the adsorbed protein results for all three surfaces are grouped together, at the interface sample the different surfaces separated out (quite markedly so in the cases of Ra (2.3) and Ra (3.8)).

The ANOVA model (Table 1) shows that the surface effects did not significantly affect the variation encountered. The vertical distribution was highly significant while the horizontal sample was not significant. Table 2 indicates that while there was undoubtedly variation between all three surfaces, no surface contributed significantly to the variation (hence the high p-value). The highly significant p-value for the vertical distribution was caused by the high values found at the interface.

5. Conclusions

The results may be summarised as follows:

a) Surface finish had a statistically significant effect on adsorbed protein (biofouling) for three out of four cases studied. This trend was not apparent for *Saccharomyces cerevisiae* which was grown and sampled under different conditions. In two cases the rougher surface (Ra (3.8)) was identified as the source of the significant variation. *Pseudomonas fluorescens* proved to be the exception to this rule with Ra (2.3) being responsible for the significant variation, even when possible interference from the vagaries of the fermentation process were removed

b) For all cases the vertical position was highly significant with regard to adsorbed protein levels assayed. This significance was due to the phase partitioning encountered (vertically) in the tank causing an increasing amount of adsorbed protein at the interface and headspace regions. It is suggested that this pattern was due to the lack of removal forces (eg. hydrodynamic shear) in the gas space.

c) In all cases the horizontal sample did not vary significantly in terms of adsorbed protein levels assayed.

d) An increase in surface roughness corresponded with an increase in the amount of adsorbed protein (biofouling) assayed in the bulk (liquid) phase and in the gas-filled headspace region of STR systems studied.

6. Acknowledgements

The authors would like to thank the DTI, National Engineering Laboratory Executive Agency who funded this work. In addition G.W. Moody (NEL), A.J.P. Spragg (NEL) and P. Davis (University of Birmingham) are gratefully acknowledged for their contributions to this work.

7. Bibliography

Arcay-Ledezma, G.J. and Slaughter, J.C. (1984). *The response of Saccharomyces cerevisiae to fermentation under carbon dioxide pressure.* J. Inst. Brew., 90. 81-84.

Hockenhull, D.J.D. (1959). *The influence of medium constituents on the biosynthesis of penicillin.* In Hockenhull, D.J.D. (ED.). Progress in Industrial Microbiology, Heywood and Co., London, 1, 3-27.

Lowry, O.H., Rosenbrough, J.H. and Farr, A.L. et al. (1951). *Protein measurement with the folin phenol reagent.* J. Biological Chemistry, 193, 265-275.

Maw, J.M. and Smith, R.N. (1986). *Monitoring microbial activity on surfaces.* In Barry, S. and Houghton, D.R. (Eds.). Biodeterioration 6. CAB International, 154-160.

Mott, I.E.C. and Bott, T.R. (1990). *The adhesion of biofilms to selected materials of construction for heat exchangers.* Ninth Int. Heat Transfer Conf., Jerusalem, Israel. 21-26.

Neish, A.C., Blackwood, A.C. and Ledingham, G.A. (1945). *Dissimilation of glucose by Bacillus subtilis (Ford's Strain).* Can. J. Res. 23B, 290-296.

Reid, D.C., Millar, R. and Bott, T.R. (1991) *A quantitative assessment of biofouling in bioprocess equipment.* Upstream and Downstream Processing in Biotechnology III, Antwerp. 67th meeting of European Fed. Biotechnology.

Timperley, D.A. (1984). *Surface finish and spray cleaning of stainless steel.* Profitability of Food Processing. Institution of Chemical Engineers Symposium Series No. 84.

Chapter 9

BIOFILM PREVENTION AND DESTRUCTION

THE DESIGN OF ANTIFOULING SURFACES: BACKGROUND AND SOME APPROACHES.

K.E. COOKSEY AND B. WIGGLESWORTH-COOKSEY
Montana State University
Bozeman, MT U.S.A. 59717

1. INTRODUCTION

In this paper we will describe some approaches that are being investigated currently with a view to preventing biofouling problems. We will focus on methods to prevent or reduce microbial film formation in the marine environment. Because of space limitations, no attempt will be made to review the biomedical implant industry. Nevertheless, some of the ideas mentioned here are applicable to that environment.

1.1. WHY DO WE NEED NEW STRATEGIES TO PREVENT BIOFOULING?

1.1.1. *Performance of Current Treatments.* The advent of the self-polishing organotin copolymers allowed the adoption of about five year intervals in painting schedules. However, although these coatings controlled animal fouling well, macroalgal fouling acceptably, they did little for what has been termed slime. Slime consists of a layer of algae (almost always a very restricted range of diatom species - Amphora, Amphiprora, Navicula, Achnanthes) and bacteria. (Dempsey, 1981; Callow, 1984, 1986; Loeb, 1984). These organisms show various levels of resistance to the toxicants of the paints (Thomas and Robinson, 1986; Callow & Evans, 1981). Although this layer is rather thin by conventional biofouling standards (c. 100μm cf. several cm), it still causes problems. Biocorrosion takes place beneath it. (Iverson, 1987), and it also causes hydrodynamic drag on a vessel. Bohlander (1992) has recently shown that the drag penalty inferred by earlier work using a laboratory device (Loeb, 1984), is in truth very large in practice. A frigate with only the microfouling removed required 18% less shaft-horsepower to achieve 26 knots. This translates to a saving of 5000 HP. Since the fuel bill for the U.S. Navy alone is of the order of $0.5 billions per year, this type of excess expenditure represents an enormous waste of resources. The time to recoup the cost of cleaning the frigate in terms of fuel saved was 22h. at 16 knots. Nevertheless, the use of fossil fuels would be even higher without the use of any antifouling coatings. The reasons why such a thin layer of microbial slime

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 529–549.

should cause so much drag are not completely understood. It is likely that the interaction of a non-rigid layer (fouling layer) with a hydrodynamic burst sweep cycle, known to exist at a smooth rigid surface, causes this increase. (Gucinski et al, 1984; Hendricks and Ladd, 1991). Unfortunately the most efficient coatings available, i.e. the alkyl tins, are now in the process of, or are already, banned from most areas of the marine world. The trialkyl tin derivatives have proved to be non-specific and are generally toxic at seawater concentrations of parts per billion. The reason for their toxicity at such a low concentration is that they are extremely non-polar and thus accumulate in the lipid tissues of all organisms in contact with seawater. Further, even where there is no outright ban on these materials, they cannot be removed from ships in dry-dock because of disposal problems. Effectively, organotin can be used no longer in marine coatings.

The loss of the organotins has been a double blow to the industry. This is because these molecules were part of the toxicant delivery system, as well as the toxicant itself. The successful coatings were co-polymers of trialkyl tin and acrylate. The bond between them hydrolyzed in seawater to release toxicant and thus produce continually, a fresh plastic surface. For this reason they were known as self-polishing paints.

1.1.2. *Alternative coatings.* The best non-alkyl tin coatings fail within three years and many of them fail after about one year. The industry is now in what we will call an interim phase, i.e. there are no revolutionary new products available, so industry is boosting the effectiveness of coatings it has available with toxicants that are registered already for agricultural use (herbicides, insecticides). Most, however, are either carbamates or highly halogenated molecules (Kjaer, 1991). This mode of operation is not likely to be a long-term solution to the problem - thus the use of the term "interim".

What may last is the current generation of paints that depend for their effectiveness on the inability of all organisms to adhere strongly to them. (Note that the word "strongly" is emphasized.) Such paints do not remain free of fouling organisms, in fact they often foul more quickly than untreated surfaces, but as the bond between the organism and the coating is weak (Rittle et al, 1990) the fouling material is comparatively easily removed. To adopt "fouling removal" rather than "prevention" as a control measure requires a large change in both procedure and philosophy on the part of those operating fleets of vessels. The new types of "non-stick" paints would be convenient for use by the small boat industry, but are not very abrasion-resistant, however. Most paint manufacturers have such coating systems available (e.g. Courtaulds: International Paint - Intersleek - FCS; Kansai: Biox; Chugoku: Bioclean). Their popular use previously was precluded by the greater success of the organotin coatings.

There are other environmental concerns involving marine coatings: Many of the proposed substitutes for alkyl tins are also environmentally dangerous because of their high halogen content; the manufacture of the chlorinated rubber base of some

marine coatings is ozone - depleting, copper has heavy-metal toxicity characteristics; the volatile organic solvents used in paint formulation contribute to air pollution and photochemically-induced smog formation. This latter problem can be solved by reformulating coatings with water, as has been done with many paints produced for use by consumers.

2. QUALIFICATION OF AN ACCEPTABLE MARINE COATINGS SYSTEM.

Having enumerated the problems with existing products it is possible to list, at least, the desirable parameters which must be considered in designing the ideal coating system.

2.1. ENVIRONMENTAL CONCERNS

The system should not cause unacceptable levels of environmental pollution in application, service or removal. Such a material would not be hazardous directly to human health.

2.2. ECONOMIC ASPECTS

Economics will play an important role. Thus costs of manufacture and testing required for government registration must be considered. Cost-benefit analysis and 2.1 will probably play the most important roles in deciding whether a new system is accepted.

2.3. COMPATIBILITY

A new system should be compatible with current technology - otherwise costs escalate enormously. A coating requiring the development of new undercoating and application systems is not likely to economically viable. Unless a new technology is revolutionary, it should also be compatible with the existing paint chemistry of the manufacturer (Kjaer, 1991). This aspect of the new system is particularly important for coatings requiring a slow-release mechanism for a new bioactive principle.

2.4. ACTIVITY SPECTRUM

The coating should be able to deal with a broad range of fouling organisms. To do this may require the combined approach of several distinct technologies. It is unlikely that the ultimate replacement for organotins will be a single molecule. At one time it was considered that the process of biofouling was an ecological succession. Surfaces placed in the sea accumulated a molecular fouling layer, bacteria, microalgae, and macrofoulers (invertebrates, macroalgae) in that obligate order. Thus, if one could keep the initial fouling layer from attaching, nothing else would

adhere to the surface. Belief in that obligate hierarchy is no longer held by workers in the field (e.g., Little, 1984). The fouling sequence is not an ecological succession and a coating must therefore deal individually with each member of the ultimate community.

2.5. LIFETIME

Since the best current technologies provide up to three years of service, an increase in this time is required. Moreover a more uniform success rate would be useful (Some coatings fail in 12-15 months). A recent draft specification from the U.S. Navy requests a lifetime of 5-7 years, depending on the application. Throughout its lifetime the coating should maintain its smooth initial finish and not, therefore, contribute to the drag of the vessel.

3. HOW DO CELLS ADHERE?

If we are to design new systems to prevent the adhesion of cells to surfaces, it is necessary to have information concerning the molecular processes involved. Since this discussion is a prelude to that in Section 5.1, only aspects of bacterial adhesion related to the surface energy of the substratum will be discussed. Other aspects of bacterial adhesion are covered elsewhere in this volume. It would have been instructive to be able to include here detailed aspects of the surface chemistry of the substrata involved in the papers cited and the means by which particular functional surface groups generated the degree of hydrophobicity (wettability). Unfortunately that type of information is only now beginning to accumulate. For instance, Gingell & Owens (1992) have measured the interaction of the slime mold Dictyostelium to Langmuir-Blodgett films of known molecular orientation. The approach to the study of cellular adhesion involving well-defined surface chemistry is also the focus of the Industry-University Center for Biosurfaces sponsored by the U.S. National Science Foundation at the State University of New York in Buffalo, U.S.A.

3.1. BACTERIA

Dexter et al (1975) was one of the first groups to measure the adhesion of natural population of bacteria to a range of surfaces placed in the ocean and correlate the extent and rates of colonization with the various critical surface tensions of the substrata. The greatest influence of the physicochemical properties of the substrata was seen early in the time-course experiments. The results showed that there was a minimum in the adhesive pattern at 25 dynes cm^{-1} ($mN.m^{-1}$) (see also figure 1). Such a surface would be hydrophobic. This paper was also one the first to try to explain the influence of substratum physicochemistry on the adhesive process when that surface was covered with a conditioning film adsorbed from the seawater (Chamberlain, 1992). Their suggestion was that in some way the film acted as a

transducer of the underlying chemistry, i.e. the molecular orientation of the adsorbate was dictated by the substratum.

Fletcher & Loeb, (1979) in controlled laboratory investigations showed in contrast that hydrophobic surfaces were rapidly colonized. Fletcher further demonstrated (McEldowney & Fletcher, 1986) that adhesion processes in four species of freshwater bacteria were dependent on the medium in which they had been grown, their growth rate, as well as their species. For example, growth in carbon-poor medium promoted attachment of *Chromobacterium*, had no effect on *Pseudomonas* and reduced adhesion in Flexibacter sp.. Generalizations were not possible. Christensen et al (1985) was able to throw some light on the confusion, at least as far as the *Pseudomonas* sp. are concerned. This group demonstrated that a marine pseudomonad grown in batch culture produced different polymers, dependent on the phase of growth. Cells attached to hydrophobic surfaces poorly during exponential growth, but a 25 fold increase in adhesion was seen when cells entered stationary phase. Attachment to hydrophilic plastic was always low and not growth-phase dependent. A completely different form of attachment polymer was formed on entry of the cells into the stationary phase. These findings go a long way to explaining some of the seemingly contradictory results in the literature.

Paul & Jeffrey (1985) showed that adhesion of *Vibrio proteolytica* to hydrophobic polystyrene was inhibited by proteolytic enzymes, but not attachment to hydrophilic plastic. This suggests that the adhesion mechanism is dependent on protein adhesive molecules in one case, but not in the other. There is an alternative explanation however. If the protein molecules were surface-active or could interfere with hydrophobic/hydrophobic interactions, they would inhibit adhesion on hydrophobic, but not hydrophilic surfaces. This explanation is favored by Rosenberg & Kjelleberg (1986).

Busscher et al (1990) worked with two strains of bacteria that cause problems in the dairy industry. A strain of *Leuconostoc* showed no clear preference in adhesion to surfaces of various surface energies, whereas a streptococcal isolate adhered to a greater extent to surfaces with high wettability (glass, polymethylmethacrylate).

It is highly likely that the adhesive properties of some of the cells used were undergoing starvation responses during the experiments mentioned above. Starvation responses start after a few minutes in substrate-free media (i.e. washed cells in buffer) and are quite apparent after two hours (Kjelleberg and Hermansson, 1984). However, once again, there appear to be no general assumptions that can be made for all species - except that cells on hydrophobic surfaces are less strongly adhered than those on hydrophilic surfaces.

A series of studies wherein a bacterium responds to an entirely different surface property in its adhesion process has been published by the group at the Agouron Institute in California (Silverman et al, 1984); Belas et al, 1986; McCarter et al, 1992). *Vibrio parahaemolyticus* is a polarly flagellate marine bacterium. When it attaches to a surface it produces a swarmer cell that possess many long peritrichous flagella. The expression of the *laf* genes responsible for initiating lateral flagella

formation has been studied by transposon mutagenesis and the inclusion of a lux sequence as a reporter gene. Thus when a vibrio cell is induced to settle, it initiates lateral flagella synthesis which causes the expression of the lux genes. The lux gene sequence contains the genetic information for the cell to become luminescent. Thus adhesive responses are translated as luminescent responses. Using this molecular biological approach, the Agouron group has found that surfaces are detected by the polar flagellum as areas of increased viscosity. Furthermore, iron limitation in the medium is required for the successful expression of the constructed genetic information. It is true to say that this vibrio possesses tactile ability that is transduced using the flagellum. Perhaps the biochemical transducer of the restriction of rotation of the flagellum in media of increased viscosity is related to the energy status of the flagellar mechanism. This work has been reviewed recently (McCarter et al, 1992). The attachment of bacteria to surfaces to produce a fouling layer appears to take place by many mechanisms that are both species and physiological state dependent. Thus it is difficult to see how this information could be used in a unique design for a surface that would remain fouling-free.

3.2. MICROALGAE

Since the majority of the organisms in the algal biomass in a slime layer on a submerged marine surface are diatoms, only the attachment mechanisms of these organism will be considered.

The work in this section was performed almost exclusively with the most commonly found marine fouling diatom, *Amphora coffeaeformis*. Adhesion of diatoms to surfaces can be followed by a simple assay. Washed cells are allowed to adhere to a glass cover slip held at an angle of 45° in a small beaker. After a period of time, usually two hours in the presence or absence of agents expected to modify the adhesion process, the glass surfaces are rinsed and cells attached determined by their chlorophyll autofluorescence signal. The washing procedure is not critical since cells are very firmly attached. Some of the information in table 1, and figures 1 & 2, was generated using this assay.

Adhesion, like motility (Cooksey & Cooksey, 1980) is calcium-dependent (Cooksey, 1981). Furthermore, it has been shown that adhesion takes place in the dark, is energy-dependent and that the source of the energy could be oxidative processes. Protein synthesis is also necessary. Note that the calcium-channel blocker, D-600 which is related to the more commonly used verapamil, also inhibits attachment. This suggests that calcium-influx is important in the adhesion process, not merely extracellular calcium. It is likely that such a system is under the regulatory control of the cell. The question now arises concerning the environmental signal that initiates the adhesive response. Adhesion is also surface-energy dependent (Fig. 1).

There have been no genetic studies concerning the means by which diatoms sense surfaces. There have been, however, studies on the physiology of the process. The chemotactic system of *A. coffeaeformis* has been used as a model to investigate

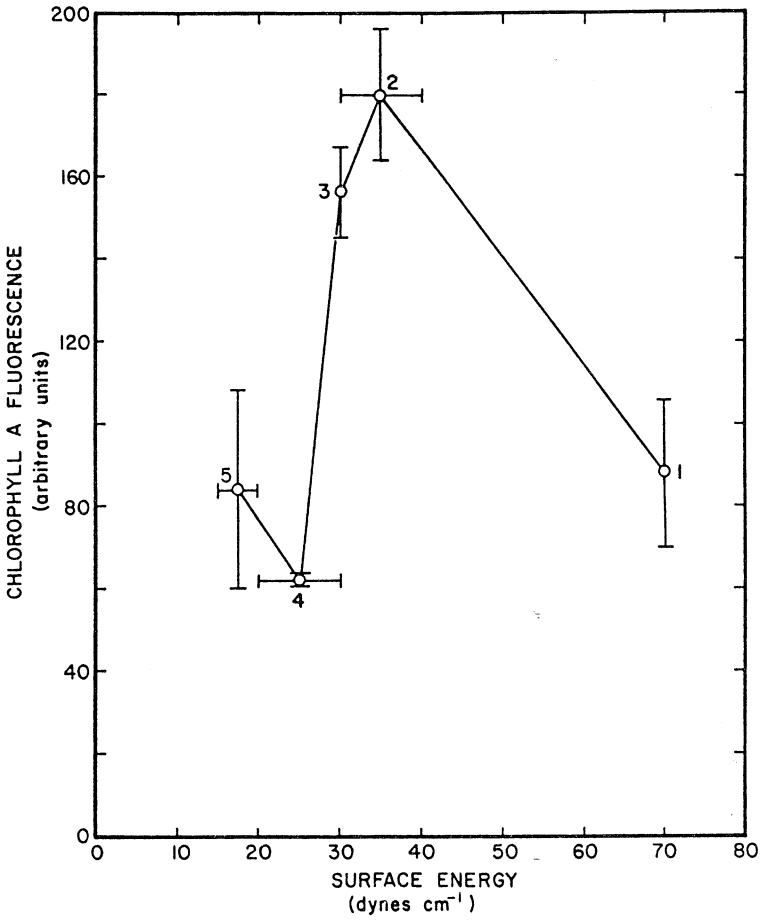


Figure 1. The relative numbers of diatoms adhered to chemically-modified glass surfaces. (Reproduced with permission from Characklis & Cooksey, 1983).

environmental transmembrane signal transfer (Cooksey & Cooksey, 1988).

Diatoms move by gliding, i.e. they cannot move unless adhered. Therefore those signals that turn on motility, especially directed motility (chemotaxis), must first initiate adhesive mechanisms. Chemotaxis is a receptor-controlled process and shows considerable specificity towards the initiating chemical signals (Cooksey & Cooksey, 1988). Figure 2 summarizes current ideas concerning the regulation of diatom adhesion. It is generally consistent with current thinking for agonist/receptor regulated processes in other cells (Berridge, 1991). An agonist, which for chemotaxis, could be a sugar related in structure to glucose, binds to a receptor (R_1) and

promotes the hydrolysis of membrane-bound phosphoinositide to inositol triphosphate (IP_3) and diacyl glycerol. Both of these are second messengers. In some cases in other types of cells, IP_3 (Tranducer T_1 in Figure 2) binds to a further receptor (R_2) which liberates calcium from a bound calcium pool (Ca-bound). This in turn opens the calcium channel and facilitates calcium-based cellular activities through an unknown number of transducers (Tn). Such a process is secretion of adhesive material contained in vesicles previously synthesized in the golgi-apparatus of the cell (Daniel et al, 1980; Webster et al, 1985).

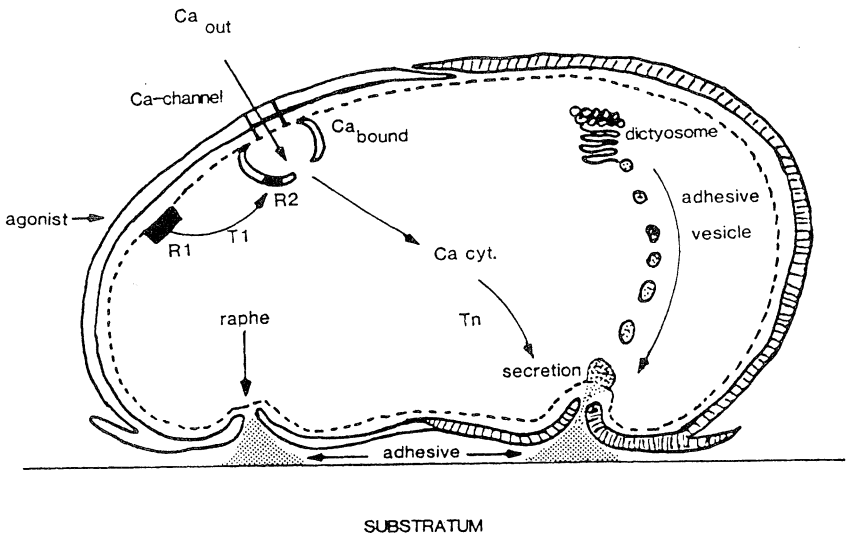


Figure 2. A model to explain diatom adhesion based on stimulus-secretion coupling. R_1 is a plasma membrane receptor. T_1 is a transducer which could be inositol triphosphate (IP_3), or a molecule that acts similarly. R_2 is another receptor which in this case binds IP_3 (or a similar molecule) leading to a release of bound calcium (Ca bound) and an increase in cytosolic calcium (Ca cyt). Tn is an unknown number of transducers that are involved in the transmission of the calcium-initiated message. The model is consistent with the calcium oscillation model proposed by Berridge (1990).

The question that now comes to mind is "What is the environmental signal that promotes adhesion? There is a little evidence that supports the idea that the marine conditioning film could be the elusive signal. Baier (1980) has suggested that the film is glycoproteinaceous and thus may consist of a protein backbone with short carbohydrate side chains. It is possible that diatoms sense the carbohydrate side chains of the conditioning film.

A more appealing idea concerns a mechanism of autosensing. Imagine that a cell synthesizes and secretes adhesive continuously, but at a very low rate. When in suspension, this material would diffuse away from the cell since it is water-soluble (Edgar & Pickett-Heaps, 1984). If a cell came in contact with a surface, diffusion would be restricted and the adhesive concentration would increase in the immediate environment of the cell. If this adhesive could react with surface-bound receptors, increased local concentrations of adhesive could derepress synthesis and secretion. This could be interpreted as either physical or chemical sensing of a surface.

It should be pointed out that the large numbers of diatoms found on surfaces submerged in the sea are likely to be the result of colonization followed by in situ growth, not of continuous recruitment. This is so even over the initial period of accumulation (48 h.) (Cooksey et al, 1984).

4. HOW DO WE INTERRUPT SPECIFICALLY THE SEQUENCE OF EVENTS LEADING TO CELLULAR ADHESION?

4.1. GENERAL MODEL OF THE ADHESIVE PROCESS

Figure 3 summarizes in a general way the events leading to adhesion and growth. It also suggests potential sites for anthropomorphic intervention.

Site 1 is the most difficult to exploit. If substratum sensing commonly involves the physical process described by the Agouron Institute Group, (section 3.1.), its specific inhibition is unlikely. It would involve changing the structure of water at an interface which would then alter the microviscosity. The relief of iron limitation would not be difficult to achieve, but since vibrio attach to steel (Stanley, 1983), this approach also is unlikely to be of use.

Exploitation of Site 2 could be used for the interruption of both diatom and invertebrate larval adhesion. If the organism senses the surface by some biochemical means, there are possibilities that the process could be confused specifically by molecules acting as repellents, by compounds that interfere with the molecular recognition process leading to a biochemical signal, or the transduction of that signal. For instance, *A. coffeaeformis* is positively chemotactic to glucose, but negatively tactic to mannose (Cooksey & Cooksey, 1988) and to extracts of the soft coral *Leptogorgia virgulata* (B.W.-Cooksey, unpublished results). Specific settling cues for invertebrate larvae have been described (Morse et al, 1979). Intervention in the fouling sequence at this level holds great promise for the future. As far as we are aware there have been no breakthroughs yet! Sites 3 & 4 are the positions at which most antifoulant

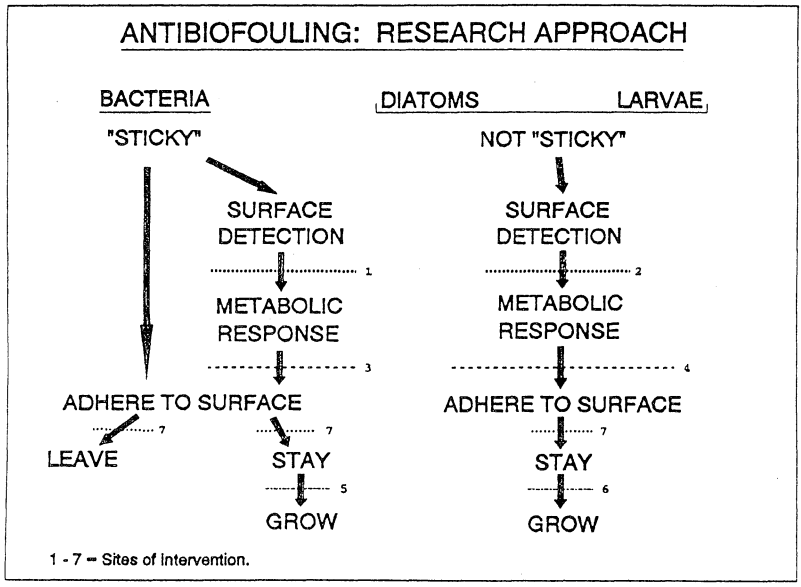


Figure 3. Potential sites for anthropogenic intervention in the sequence of events in biofouling. Dotted horizontal lines identify the processes where intervention does, or could, take place. The time scale, which runs vertically on the figure is not linear. The diagram assumes that all organisms reach the surface by hydrodynamic means.

molecules have operated in the past, i.e. they are general metabolic poisons, often heavy metals. There is little biological specificity available at this level. Lack of specificity can be regarded either as an economic advantage, or as an environmental disadvantage.

The process of growth is an integration of all metabolic steps. It is possible to interfere with the progress of events at any level, but a molecule that is active here will be, by definition, a general poison (Sites 5 & 6). New antifoulant molecules should not be sought among compounds of this kind, unless they are extraordinarily short-lived in the environment and steep concentration gradients can be maintained from the coating to the overlying water. Site 7 refers to the surfaces that facilitate the release of adhered fouling organisms. They are often referred to as "non-stick" coatings. The flow chart indicates that this strategy is a general one. It is well known

that coatings of low surface energy can fulfill this role. Once the physicochemical bond between the surface is established, biology has little more to do in the adhesive process. The use of this type of coating assumes many of the interactions between cell and surface are promoted by hydrophobic interactions. While such interactions are common, interactions based on surface charges also exist.

It seems likely therefore that no one strategy of coating design will be universally acceptable. The use of fouling release coatings implies that vessels will require cleaning. For small craft this can be accomplished with a water jet after their removal from the water. The U.S. Navy, in draft specifications for antifouling coatings, has suggested that water delivered at 2000 psi for 2 min. should be sufficient to remove fouling organisms (This can be achieved with a fire-hose). Paint manufacturers have suggested a much less vigorous cleaning is necessary. Large vessels must be cleaned in situ with diver-controlled or remotely operated vehicles equipped with brushes or ultrasonic transducers.

5. SEARCH FOR NEW ANTIFOULANT MOLECULES

The discovery process, either by synthesis or search, should be guided by biological assays that are relevant to the problem. For instance, they should be based on some significant metabolic process in appropriate fouling organisms. In other words the antibiofouling industry should adopt a procedure similar to that used to find new drugs.

5.1. FOULING RELEASE COATINGS

The problems in increasing the abrasion resistance of coatings of this type will be solved in the organic chemical synthesis laboratory. It has been suggested that bleeding of silicones into the environment from fouling release surfaces should be avoided (R.E. Baier, personal communication). To solve this problem, Baier has proposed the use of polyether type polyurethane films to which are covalently tethered polydimethyl-siloxanes (Gucinski et al, 1984; Hendricks & Ladd, 1991). Some laboratories are trying fluorinated urethanes as fouling release agents. In making new molecules, a balance has to be struck between extremely hydrophobic surfaces which foul quickly, but clean easily, and the reverse situation. The existence of the so-called "biocompatibility zone" around 25 mNm^{-1} will guide this work.

Bioassays for fouling release coatings must encompass some kind of regulated distractive force that can be applied to the attached organisms. The flow cell as described by Fowler is useful (Fowler & McKay, 1980), especially if the modified mathematics proposed by Fryer et al (1985) are followed. Rittschoff et al (1992) have described the use of a regulated jet of seawater produced by a device similar to that used to clean teeth. More sophisticated devices to measure colonization of surfaces under regulated flow regimes have been described by Lawrence et al (1989) and Sjollema et al (1988).

5.2. INHIBITORY MOLECULES

5.2.1. *Types of Inhibitory Molecules.* The aim of this section is to suggest strategies to discover antibiofoulant molecules that are not generally toxic. A further goal is to suggest means of screening for molecules that have specific modes of action. Specificity in biological systems is determined by schemes involving molecular recognition followed by transduction of the recognition signal. Often this entails a time- dependent change in intracellular calcium concentration (Berridge, 1991). The initial recognition site for the signal is the cell membrane and involves occupancy of a receptor. The three-dimensional structure of the receptor protein and that of the extracellular signalling molecule (agonist) are the means for controlling specificity. If the biochemistry of the interaction between the receptor and the agonist is known, it should be possible to design compounds that will mimic the agonist, but will not give rise to the normal cascade of events resulting from the receptor binding process. In some cases this could be invertebrate larval metamorphosis. An example from mammalian biology will make this clearer. Mammalian cells adhere to surface to which certain proteins are adsorbed. These proteins have a common tripeptide amino acid sequence to which the adhering cells respond. The sequence of amino acids is arginine-glycine-aspartate which is usually abbreviated as RGD. Cells are prevented from adhering in the presence of soluble RGD peptides because surface bound and soluble RGD sequences compete for the same receptor on the cell. Further the RGD sequence can be bound covalently to plastic surfaces making that surface able to be involved in specific cellular adhesion (Massin & Hubbell, 1990). The production of an analogue of the RGD sequence, say one containing a D-amino acid rather than an L-form, may prevent adhesion. Although this type of technology has been used to design biocompatible surfaces for bioimplantation devices, I do not believe it has been used to prepare bioincompatible surfaces. This approach has not been used at all in the marine environment.

A speculative example of how this may work is as follows. Spawning of mussels and sea urchins is triggered by the availability of phytoplankton food. Starr et al (1990) showed that a heat-stable molecule from marine diatoms (including *Phaeodactylum tricornutum* and *Skeletonema costatum*) promoted spawning in sea urchins and mussels. An analogue of the spawning promoter, or even part of the sequence if a peptide (cf. RGD-sequence), could by the same token prevent spawning. Thus in screening for bioactive molecules it is as well to search for those that promote, as well as inhibit, the process of biofouling.

5.2.2. *Sources of Bioactive Molecules.* Some sources have been mentioned above. The almost classical place to search for bioactive molecules is marine invertebrates (Faulkner, 1984). In one report (Thompson et al, 1985), 38 bioactive natural products were isolated from a survey of 40 sponges. Bioassays for antibacterials, invertebrate settlement and metamorphosis activity were performed. Many of the compounds were sequiterpenes, but some were derived from dibromotyrosine. Two

of these later compounds (aerothionin and homoaerothionin) were exuded into seawater continuously, and thus may act as natural slowly-released antifoulant molecules for the organism (Thompson, 1985). In this case nature seems to have designed the molecule and its means of delivery to the environment. Perhaps an approach where potential antifoulant molecules are extracted from the aqueous environment of an animal may be more successful than the extraction of a homogenate of the whole animal.

A group at Duke University Marine Laboratory has also been working with soft coral extracts using barnacle cyprid and bryozoan settling assays. They have found that methanolic extracts of Leptogorgia virgulata and Renilla reinformis are active antibiofoulants. The active principle of these extracts is a family of molecules of which renillafoulin (figure 4) is an example. The structure of this molecule is the subject of a patent (Costlow et al, 1988). A chemical company has agreed to exploit analogues of this molecule for antibiofouling use. Some details of this work should be available by mid-1992 when expanded patent protection has been obtained.

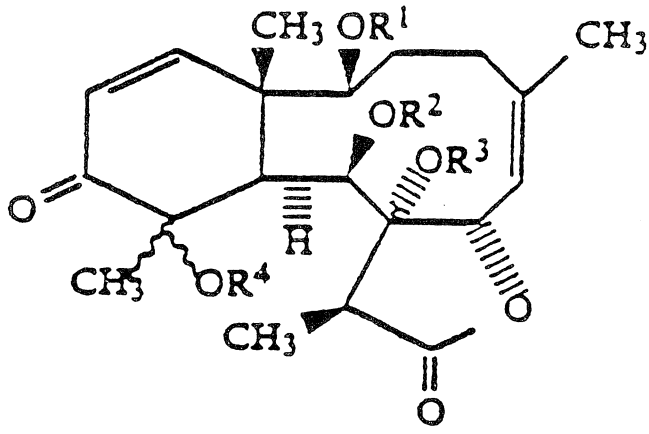


Figure 4. The structure of renillafoulin. R_1 - R_4 represent H or a C_1 - C_{18} alkanoyl or alkenoyl group.

Other organisms using chemical defense strategies include nudibranchs (Cimino et al, 1983), cyanobacteria (Bagchi et al, 1990) and tropical woods (Jurd & Mannens, 1978). The press, (Rensburger, 1992; Stone, 1992) has recently become aware of research on tropical trees as a potential panacea. Significantly, the Neem tree is mentioned as a source of bioactive materials that prevent tooth decay. This may be exploitable in terms of antibiofoulant activity. Products from the tree, such as azadirachtin, are already formulated as insecticides. If their action in insects is analogous to that in other invertebrates, they may be potential inhibitors of marine exoskeleton molting. The use of extracts of Neem tree leaves was described in Sanskrit writings of a 1000 years ago! (Rosenberg, 1992).

5.2.3. *Laboratory Bioassays.* Assays to measure the dynamics of the adhesion of bacteria to surfaces have been described in detail (e.g. Lawrence et al, 1989; Sjollema et al, 1988, 1989). Some controversy concerns the necessity to use a dynamic system (Bryers, 1992), since much of our information concerning the adhesion of bacteria to surfaces has been obtained using static systems (see Fletcher, 1992). Diatoms have been largely neglected as indicator organisms for the selection of antibiofouling strategies. This is in spite of the fact that almost every illuminated surface in the marine environment rapidly become colonized with them. Callow et al (1986) have described a bioassay procedure similar to that of Cooksey (1981). Diatoms are allowed to attach to attach to an experimental surface and their number is determined from an estimation of surface bound chlorophyll a or ATP. Milne & Callow (1985) have also used the Fowler flow cell (Fowler & McKay, 1980).

Table I shows the influence of various biochemical inhibitors on the motility and adhesion of *A. coffeaeformis* (Cooksey, 1981; Cooksey & Cooksey, 1980, 1986). Motility assays are performed after incubating cells with the desired inhibitor, however, microscopic counts of motility are tedious.

Recently, we have discovered that *A. coffeaeformis* is capable of directed motility in chemical concentration gradients and have proposed this phenomenon as a model for transmembrane signalling in diatoms (Cooksey & Cooksey, 1988). Compounds that interfere with transmembrane signals that result in the secretion of polymers involved in adhesion (or motility), are potential antifoulants. Note that extracts of *L. virgulata* and *Pseudoterogorgia americana* caused negative taxis (*P. a.*) and cessation of movement (*P. a.* and *L. v.*). These assays are also very tedious since they depend on the manual measurement of the number of cells treated, the number of motile cells, and the angle made by motile cells to a line drawn parallel to the concentration gradient of the extract. The diatom cells were exposed to a gradient of bioactive material in a chamber designed to measure leukocyte chemotaxis (Zigmond, 1977). It is possible to automate the analysis using commercial software and an image analysis system (Wigglesworth-Cooksey & Cooksey, 1991).

5.2.4. *Determination of Necessary Release Rates for Coatings.* Most bioassays are designed to measure biological response at one concentration of active material at a time. For an antifouling system to be practical in the environment, the active principle of the coating must be released at a constant rate over the life of the coating. Before the coating technology can be designed effectively to achieve this, target release rates must be known. These can be determined by allowing active materials to diffuse into an open system from one side of a porous membrane, the alternate side of which is bathed in a solution of the active material. Biofouling of the open side of the membrane can be determined (Houghton, 1984).

5.2.5. *Formulation.* The subject of formulation of bioactive materials into coatings is too complex to treat here. It is also an area of technology that is closely guarded by coatings companies. It seems likely that microencapsulation of active materials may

Table 1. Compounds Affecting Adhesion and Motility.

Compound Concentration	Motility	Adhesion	Metabolic Process Affected
DCMU, 2μM	0	0	Photosynthesis, P.S.II
CCCP, 1.25μM	-	-	All energy generation
Cycloheximide, 3.6μM	NT	-	Protein synthesis
Darkness	0	0	Photosynthesis
Tunicamycin 0.5μg ml^{-1}	-	-	Glycoprotein synthesis
Cytochalasins D,E 25μg ml^{-1}	-	NT	Actin-based cytoskeletal activity
Colchicine 25μg ml^{-1}	0	NT	Tubulin-based cytoskeletal-activity
Podophyllotoxin 25μg ml^{-1}	-	0	Tubulin-based cytoskeletal-activity
Reduced Ca^{2+}, 0.25mM	-	-	Various metabolic processes, e.g. secretion
Sr^{2+} 2.5mM	-	0	Potential Ca^{2+} replacement
Ruthenium Red, 6μM	-	NT	Transmembrane Ca^{2+} flux
D-600	-	-	Intracellular Ca^{2+} flux
Extracts of Leptogorgia virgulata[b], 50mg ml^{-1}	-	-[a]	unknown
Extracts of Pseudopter-ogorgia americana[b], 5mg ml^{-1}	-	-	unknown

- Inhibits motility or adhesion
0 Has no effect on motility or adhesion
NT Not tested

[a] unpublished work, B. Wigglesworth-Cooksey
[b] Extracts from Nancy McKeever Targett, U. of Delaware

play a role in the formulation of coatings in the future, especially if dual approaches to antifouling were used, e.g. a fouling release coating with integral bioactive materials. An innovative, although at the moment very expensive, release system has been described by Price et al (1992). In this system copper microtubules (0.2-0.4μm in diameter and up to 250μm in length) formed on a phospholipid template (Schnurr et al, 1990) are used to contain bioactive materials. They have been formulated into commercial type coatings and have been shown to be effective slow release agents (Price et al, 1992). The tubules act by slowly leaching the bioactive material from the lumen as well as by the gradual degradation of the tubule itself. The release rates are linear. Tetracycline, isothiazolone and renillafoulin have been used as bioactive materials in these microtubules contained in commercially-available coatings.

5.2.6. *Field Assays.* The most common are well known and consist of placing tiles approximately 30cm x 30cm in the marine environment and assessing fouling with time. Scientists at the Duke University Marine Laboratory have devised a system which requires a much smaller amount of coating material. This system is excellent for transitioning a prototype coating from the laboratory to the field for testing. Fiberglass rods (110 x 7mm) are coated with the paint formulation, dried, and hung from polypropylene fish netting in the sea. The rods are inexpensive and fouling on them can be quantified easily. Large numbers of rods can be exposed allowing much better statistics (larger n) than conventional fouling tiles (Rittschoff et al, 1986). Furthermore, because the rods are circular in cross-section, there is no "edge effect".

5.2.7. *Problems that may be encountered with New Coatings.* All new bioactive materials for coatings are likely to be tested for toxicity and fate by various national governmental pollution prevention agencies, even though the materials, or their close relatives, are actually natural products. A fouling release coating that does not bleed components into the environment will be easier to license.

The most common problem that natural products will encounter is one of cost. Continuous extraction from nature is not an answer. Synthesis is usually too costly, except for simple molecules. Biotechnological synthesis is, however, a strong possibility for complicated molecules. A further alternative is the chemical synthesis of biomimetic molecules that are simpler in structure than those extracted from whole organisms. At least one company has chosen this route to the production of molecules that are economically competitive. It is our opinion that using natural molecules to inspire organic chemical synthesis is the most likely route to environmentally-acceptable molecules for biofouling prevention.

6. CONCLUSIONS

At no time in its history has the antibiofouling industry depended so greatly on the findings of basic molecular science. The future holds the promise of coatings formulated with specific bioactive materials that are not only effective, but are environmentally innoxious. The products will, however, be more expensive than the materials we are using today. They are likely to depend on more than one antifouling strategy.

7. ACKNOWLEDGEMENT

We thank the U.S. Office of Naval Research for the support of work in our laboratory and Karen Bess for the preparation of the manuscript.

8. REFERENCES

Bagchi, S.N., Palod, A. and Chauhan, V.S. (1990) "Algicidal properties of a bloom-forming blue-green alga, *Oscillatoria* sp.", J. Basic Microbiol. 30, 21-29.

Baier, R.E. (1980) "Substrata influences on the adhesion of microorganisms and their resultant new surface properties", in G. Bitton & K. Marshall (eds.), Adsorption of Microorganisms to Surfaces. Wiley, New York, pp. 59-104.

Belas, R., Simon, M. and Silverman, M. (1986) "Regulation of lateral flagella gene transcription in *Vibrio parahaemolyticus*", J. Bacteriol. 167, 210-218.

Berridge, M.J. (1991) "Cytoplasmic calcium oscillations: A two pool model", Cell Calcium 12, 63-72.

Bohlander, G.S. (1992) "Biofilm effects on drag: Measurements on ships", Trans. I. Mar. E. (c) 103, Conf. 3, 16.1-16.4.

Bryers, J.D. and Characklis, W.G. (1992) "Biofilm Laboratory Methods: The use of flow reactors" in Melo et al (eds.), Biofilms: Science and Technology. Kluwer Academic Publishers, Dordrecht, The Netherlands, this volume.

Busscher, H.J., Bellon-Foutaine, M-N., Mozes, N., Van der Mei, H.C., Sjollema, J., Cerf, O. and Rouxhet, P.G. (1990) "Deposition of *Leuconostoc mesenteroides* and *Streptococcus thermophilus* to solid substrata in a parallel plate flow cell", Biofouling 2, 55-74.

Callow, M.E. (1984) "Fouling algae from "in-service" ships", Botan. Mar. 29, 351-357.

Callow, M.E. (1986) "A world-wide survey of slime formation on anti-fouling paints, in L.V. Evans and K.D. Hoagland (eds.), Algal Biofouling, Elsevier, Amsterdam, pp. 1-20.

Callow, M.E. and Evans, L.V. (1981) "Some effects of triphenyltin chloride on *Achnanthes subsessilis*, Botan. Mar. 24, 201-205.

Callow, M.E., Pitchers, R.A. and Milne, A. (1986) "The control of fouling by non-biocidal systems" in L.V. Evans and K.D. Hoagland (eds.) Algal Biofouling, Elsevier, Amsterdam. pp. 145-158.

Chamberlain, A.H.L. (1992) "Role of adsorbed layers in bacterial adhesion" in Melo et al (eds.), Biofilms: Science and Technology, Kluwer Academic Publishers, Dordrecht, The Netherlands, this volume.

Characklis, W.G. and Cooksey, K.E. (1983) "Biofilms and microbial fouling" in A.I. Laskin (ed.), Applied Microbiology, 29, Academic Press, New York, pp. 93-138.

Christensen, B.E., Kjosbakken, J. and Smidsrod, O. (1985) "Partial chemical and physical characterization of two extracellular polysaccharides produced by marine, periphytic *Pseudomonas* sp. strain NCMB 2021", Appl. Environ. Microbiol. 50, 837-845.

Cimino, G., DeRosa, S., DeStefano, S., Sodano, G., Villani, G. (1983) "Dorid nudibranch elaborates its own chemical defense", Science, 219, 1237-1238.

Cooksey, B. and Cooksey, K.E. (1980) "Calcium is necessary for motility in the diatom *Amphora coffeaeformis*", Plant Physiol. 65, 129-131.

Cooksey, B. and Cooksey, K.E. (1988) "Chemical signal response in diatoms of the genus *Amphora*, J. Cell Science, 91, 523-529.

Cooksey, B, Cooksey, K.E., Miller, C.A., Paul, J.H., Rubin, R.W. and Webster, D. (1984) "The attachment of microfouling diatoms" in J.D. Costlow and R.C. Tipper, (eds.), Marine Biodeterioration: An Interdisciplinary Study, U.S. Naval Institute Press, Annapolis, MD., pp. 167-171.

Cooksey, K.E. (1981) "Requirement of calcium in adhesion of a fouling diatom to glass", Appl. Environ. Microbiol. 41, 1378-1382.

Costlow, J.D., Hooper, I.R. and Rittschoff, D. (1988) U.S. patent No. 4,788,302.

Daniel, F.G., Chamberlain, A.H.L. and Jones, E.B.G. (1980) "Ultrastructural observations on the marine fouling diatom *Amphora*", Helgolander meeresunters. 34, 123-129.

Dempsey, M.J. (1981) "Colonization of antifouling paints by marine bacteria", Botan. Mar. 24, 185-191.

Dexter, S.C., Sullivan, J.D., Williams, J. and Watson, S.W. (1975) "Influence of substrate wettability on the attachment of marine bacteria to various surfaces", Appl. Microbiol. 30, 298-308.

Edgar, L.A. and Pickett-Heaps, J.D. (1984) "Diatom locomotion" in F.E. Round & D.J. Chapman (eds.), Progress in Phycological Research 3, 47-88.

Faulkner, D.J. (1984) "Marine natural products: Metalolites of marine invertebrates", Natural Product Rep. Roy. Soc. Chem. (Lond.) 1, 551-598.

Fletcher, M. (1992) "The measurement of bacterial attachment to surfaces in static systems" in Melo et al (eds.), Biofilms: Science and Technology, Kluwer Academic Publishers, Dordrecht, The Netherlands, this volume.

Fletcher, M. and Loeb, G.I. (1979) "Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces, Appl. Environ. Microbiol. 37, 67-72.

Fowler, H.W. and McKay, A.J. (1980) "The measurement of microbial adhesion" in R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter and B. Vincent (eds.), Microbial Adhesion to Surfaces, Ellis-Horwood, Chichester, pp. 143-161.

Fryer, P.J., Slater, N.K.H. and Dudderidge, J.E. (1985) "Suggestions for the operation of radial flow cells in cell adhesion and biofouling studies", Biotech. Bioeng. 27, 434-438.

Gingell, D. and Owens, N.F. (1992) "Adhesion of Dictyostelium amoebae to deposited Langmuir-Blodgett monolayers and derivatized solid surfaces. A study of the conditions which trigger a contact-mediated cytoplasmic contractile response", Biofouling 5, 205-227.

Gucinski, H., Baier, R.E., Meyer, A.E., Fornalik, M.S. and King, R.W. (1984) "Surface microlayer properties affecting drag phenomena in seawater", in Sixth International Congress on Marine Corrosion and Fouling, August, 1984, Athens, pp. 585-604.

Hendricks, E.W. and Ladd, D.M. (1991) "Skin friction measurements in flows over tethered polymer coatings", Naval Ocean Systems Center, San Diego, CA, Technical Report. 1438, pp. 1-7.

Houghton, D.R. (1984) "Toxicity testing of candidate antifouling agents and accelerated antifouling paint testing", in J.D. Costlow and R.C. Tipper (eds.), Marine Biodeterioration: An Interdisciplinary study, U.S. Naval Institute Press, Annapolis, MD., pp. 254-258.

Iverson, V.P. (1987) "Microbial corrosion of metals", Adv. Appl. Microbiol. 32, 1-36.

Jurd, L. and Manners G.D. (1976) "Biocides from marine borer resistant timbers", in J.D. Bultman (ed.) Proc. Workshop on the Biodeterioration of Tropical Woods, U.S. Naval Research Laboratory, Washington, D.C., pp. 7-14.

Kjaer, E.B. (1991) "Bioactive materials for antifouling coatings", Paper presented at the 17th Int. Conf. in Organic Coatings Science & Technology, Lucerne, Switzerland, August, 1991.

Kjelleberg, S. and Hermansson, M. (1984) "Starvation-induced effects on bacterial surface characteristics", Appl. Environ. Microbial. 48, 497-503.

Lawrence, J.R., Korber, D.R. and Caldwell, D.E. (1989) "Computer-enhanced darkfield microscopy for the quantitative analysis of bacterial growth and behavior on surfaces. J. Microbiol. Methods 10, 123-138.

Little, B.J. (1984) "Succession in microfouling", in J.D. Costlow and R.C. Tipper (eds.), Marine Biodeterioration: An Interdisciplinary Study, U.S. Naval Institute Press, Annapolis, MD., pp. 63-74.

Loeb, G.I., Laster, D. and Gracik, T. (1984) "The influence of microbial fouling films on hydrodynamic drag of rotating discs", in J.D. Costlow and R.C. Tipper (eds.), Marine Biodeterioration: An Interdisciplinary Study, U.S. Naval Institute Press, Annapolis, MD., pp. 88-100.

Massin S.P. and Hubbell J.A. (1990) "Covalently-attached GRGD on polymer surfaces promotes biospecific adhesion of mammalian cells", Ann. N.Y. Acad. Sci. 589, 261-270.

McCarter, L.L., Showalter, R.E. and Silverman, M.R. (1992) "Genetic analysis of surface sensing in *Vibrio parahaemolyticus*, Biofouling 5, 163-176.

McEldowney, S. and Fletcher, M. (1986) "Effect of growth conditions and surface characteristics of aquatic bacteria on their attachment to solid surfaces", J. Gen. Microbiol. 132, 513-523.

Milne, A. and Callow, M.E. (1985) "Non-biocidal antifouling processes", in Polymers in the marine environment, Trans. I. Mar. E. (c) pp. 229-233.

Morse, D.E., Hooker, N., Duncan, H. and Jensen, L. (1979) "Gamma-amino butyric acid, a neurotransmitter, induces planktonic abalone larvae to settle and begin metamorphosis", Science, 204, 407-410.

Paul, J.H. and Jeffrey, W.H. (1985) "Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic surfaces in *Vibrio proteolytica*", Appl. Environ. Microbiol. 50, 431-437.

Price, R., Patchan, M., Clare A., Bonaventura, J. and Rittschoff, D. (1992) "Elimination of rosin from vinyl antifouling coatings by the use of novel cylindrical channel-forming microtubules", Trans. I. Mar. E. (c) 103 Conf. 3, 18.1-18.7.

Rensburger, B. (1992) "Finding the neems to an end", Washington Post (weekly edit.), February 24, p. 38.

Rittle, K.H., Helmstetter, C.E., Meyer, A.E. and Baier, R.E. (1990) "*Escherichia coli* retention on solid surfaces as functions of substratum surface energy and growth phase", Biofouling 2, 121-130.

Rittschoff, D., Hooper, I.R. and Costlow, J.D. (1986) "Barnacle settlement inhibitors from sea pansies, *Renilla reniformis*", Bull. Mar. Sci. 39, 376-382.

Rittschoff, D., Clare, A.S., Gerhart, D.J., Bonaventura, D., Smith, C., and Hadfield, M.G. (1992) "Rapid field assessment of antifouling and foul-release coatings. Biofouling, in press.

Rosenberg, M. and Kjelleberg, S. (1986) "Hydrophobic interactions: Role in bacterial adhesion", in K.C. Marshall (ed.), Advances in Microbial Ecology, 9, Plenum Press, New York, pp. 353-393.

Schnurr, J.M., Schoen, P.E., Yager, P., Calvert, J.M., Georger, J.H. and Price, R. (1990) "Metal clad lipid structures", U.S. Patent No. 4,911,981.

Silverman, M., Belas, R. and Simon, M. (1984) "Genetic control of bacterial adhesion", in K.C. Marshall (ed.), Microbial Adhesion and Aggregation, Springer-Verlag, Berlin, pp. 95-107.

Sjollema, J., Busscher, H.J., and Weerkamp, A.H. (1988) "Deposition of oral streptococci and polystyrene latices onto glass in a parallel plate flow cell. Biofouling 1, 101-112.

Sjollema, J., Busscher, H.J., and Weerkamp, A.H. (1989) "Real-time enumeration of adhering organisms in a parallel plate flow cell using automated image analysis", J. Microbiol. Methods 9, 73-78.

Stanley, P.M. (1983) "Factors affecting the irreversible attachment of *Pseudomonas aeruginosa* to stainless steel", Can. J. Microbiol. 29, 1493-1499.

Starr, M., Himmelman, J.H. and Therriault, J.-C. (1990) "Direct coupling of marine invertebrate spawning with phytoplankton blooms", Science 247, 1071-1074.

Stone, R. (1992) "A biopesticidal tree begins to blossom", Science 255, 1070-1071.

Thomas, T.E. and Robinson, M.E. (1986) "The physiological effects of the leachates from a self-polishing organotin antifouling paint on marine diatoms", Mar. Env. Res. 18, 215-229.

Thompson, J.E. (1985) "Exudation of biologically-active metabolites in the sponge *Aplysinia fistularis* 1. Biological evidence", Marine Biol. 88, 23-26.

Thompson, J.E., Walker, R.P. and Faulkner, D.J. (1985) "Screening and bioassays for biologically-active substances from forty marine sponge species from San Diego, California, U.S.A.", Marine Biol. 88, 11-21.

Webster, D.R., Cooksey, K.E. and Rubin, R.W. (1985) "An investigation of the involvement of cytoskeletal structures and secretion in gliding motility of the marine diatom *Amphora coffeaeformis*", Cell Motility 5, 103-122.

Wigglesworth-Cooksey, B. and Cooksey, K.E. (1991) "Image analysis and chemical anti-foulants. Poster presented at International Marine Biotechnology Conf., Baltimore, October, 1991.

Zigmond, S. (1977) "Ability of polymorphonuclear leukocytes to orient to gradients of chemotactic factors", J. Cell Biol. 75, 606-616.

DEPOSITION OF CALCIUM CARBONATE WITHIN ALGAL BIOFILMS ON ANTIFOULING PAINTS IN HARD WATERS

CAROLYN R. HEATH, MAUREEN E. CALLOW and B.S.C. LEADBEATER
School of Biological Sciences
The University of Birmingham,
Birmingham, B15 2TT, United Kingdom

1. Introduction

The major types of biofouling in freshwater appear to be due either to green macroalgae (e.g. *Cladophora, Ulothrix, Enteromorpha)* or slime (particularly diatoms and cyanobacteria) (see Evans & Hoagland, 1986). Current antifouling coatings control weed and slime fouling reasonably well but a more serious problem encountered in hard waters is caused by the deposition of calcium salts. Calcareous deposits can form a hard continuous crust up to several millimetres thick on the bottom of boats painted with antifouling coatings. Areas of greatest commercial importance where calcification occurs include many rivers and lakes in Europe e.g. Lake Constance. Areas in Britain where this problem is known to be particularly severe include the Norfolk Broads, parts of the River Ouse and a number of marinas. Calcareous deposits on boats and other structures are unsightly and difficult to remove. Furthermore, cleaning often causes damage to the underlying substrate. None of the current range of antifouling coatings satisfactorily controls calcification since all attract the deposit, albeit to varying degrees. In general, paints containing algicides attract the least whilst paints based on other biocides and non-toxic "non-stick" coatings attract the most deposit.

Hard water chemistry is summarised in fig. 1. Carbon dioxide reacts with water to form weak carbonic acid which dissociates into bicarbonate and carbonate ions. Equilibrium between bicarbonate and carbonate is almost instantaneous, while the hydration of carbon dioxide takes longer (Borowitzka, 1982). The pH of the water determines speciation and at values higher than pH 8.0, as seen in hardwaters, bicarbonate is the predominant carbon source. Calcium ions are washed into the hardwater systems and react with carbonate. The solubility product of both ions is low and if exceeded, precipitation of solid calcium carbonate occurs.

Precipitation of calcium carbonate by macrophytes and eukaryotic macroalgae in hard, calcium-rich, fresh waters is a well known phenomenon in which photosynthesis plays a key role (Borowitzka, 1982). Of wider distribution and relevant to biofouling of surfaces, is calcification

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 551–556.

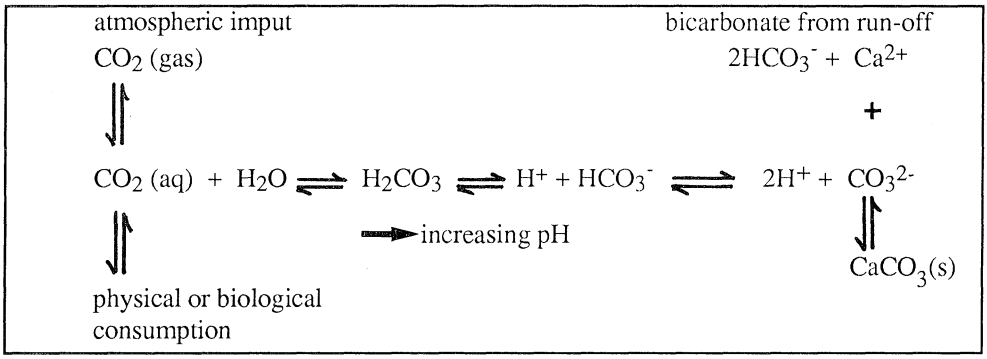


Fig. 1. Principles of hard water chemistry.

by microorganisms particularly cyanobacteria, in which photosynthesis probably plays little part. Three general requirements must be satisfied for mineral deposition to occur (1) the microenvironment in which deposition occurs must be diffusion limited (2) modification of at least one ion must occur at or near the deposition site (3) an interface must be present for mediating and possibly controlling crystal growth (Simkiss, 1986). For the deposition of calcium carbonate by microalgae, photosynthetic fixation of CO_2 leads to the removal of CO_2 from the medium with the consequent rise in pH, and eventually results in the precipitation of $CaCO_3$ on a suitable nucleating site (House, 1984). Mineral deposition can be inhibited or prevented by critically altering one of the above factors. For instance, nucleation and crystal growth can be inhibited by the addition of ortho- or polyphosphates or other crystal poisons.

In cyanobacteria and other microorganisms calcification arises from nucleation of $CaCO_3$ upon or within the extracellular polysaccharide sheath and several cyanobacteria have been shown to exhibit specificity for calcification. In addition to calcification, the sheath can trap sediment particles which then become bound into the cyanobacterial mat as growth continues. Stromatolites are formed in this way and calcified paint surfaces can be regarded as flat stromatolites. Although biomineralization processes are thought to be responsible for calcification of antifouling paints it is possible that in certain circumstances inorganic particles or even calcite crystals could act as nucleating sites.

The present investigation is aimed at establishing to what extent calcareous deposits on antifouling surfaces are biologically mediated and to ascertain which organisms are involved.

2. Material and Methods

Alkalinity and calcium were measured following the methods of Makereth *et al.*, (1978) and Grimshaw *et al.*, (1989) respectively. The saturation index (SI) was calculated according to Kelts & Hsu, (1978). Panels (8x10cm) painted with either a commercial antifouling paint or a non-biocidal control paint were immersed for six months from May 1991 in a marina at Ely, Cambridgeshire, UK. Panels were arranged on boards at five depths from the surface of the water; depth 1=25cm, depth 2=35cm, depth 3=45cm, depth 4=55cm, depth 5=65cm. There were 4 boards, each with 30 panels arranged randomly, allowing four panels of each treatment from each depth to be removed 8, 16 and 24 weeks after immersion (Heath *et al.*, submitted for publication) Accumulation of fouling was assessed by removal of standard areas of biofilm from the panels which were analysed as follows: Chlorophyll was extracted in DMSO by the method of

Jeffery & Humphrey, (1975). Dry weight (W1) was determined after drying at 90°C for 24h. The dried samples were then treated with excess 0.2N HCl to dissolve $CaCO_3$ and redried after removal of the acid. The residue (inorganic + organic) was weighed (W2) after drying at 90°C for 24h. The weight of $CaCO_3$ was calculated from the determined weights i.e.W1-W2.
Axenic cultures of algae isolated from panels immersed in Ely marina were subcultured into 250ml conical flasks containing 100ml of an artficial medium of similar composition to that found in hard waters, with a SI of 1.34. Control flasks did not contain algae. Flasks were incubated at 24°C on a shaker under continuous illumination. 5ml aliquots from each of three replicate flasks were removed every 2d for pH, soluble Ca and chlorophyll determinations.

3. Results

Microscopic examination of fresh biofilms revealed a predominance of algae. The dominant types were filamentous and unicellular green algae and diatoms. The species distribution was similar at all depths, although there appeared to be a smaller proportion of green algae at the two lower depths and more diatoms. A transverse section through a biofilm growing on a non-biocidal paint shows heavy deposition of $CaCO_3$ within the biofilm (fig.2). X-ray microanalysis confirms the crystalline material is a calcium salt. The absence of any other major peaks indicates that Ca is most likely to be present as $CaCO_3$.

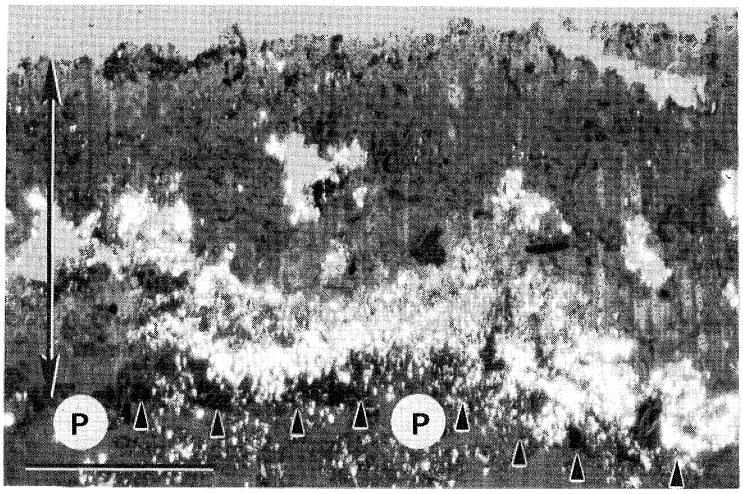


Fig. 2. Photograph of transverse section through a calcified biofilm observed with polarizing filters showing paint (P), paint/biofilm interface (arrowheads), thickness of biofilm (between arrows) and white refractive granules of calcium carbonate. Scale bar=50μm.

The composition of the water in the Ely marina is shown in Table 1. The highest SI value of 2.0 was recorded in July. All measurements were recorded at two depths viz.10cm and 60cm below the surface but since no differences were found all values were used in the means. Crystals of $CaCO_3$ were not found in the water samples.

Ca (mg L^{-1})	116.80
conductivity ($\mu S cm^{-1}$)	828.20
pH	8.54
alkalinity (mEq L^{-1})	7.07
PCO_2 (atmos)	$1.39x10^{-3}$
SI (saturation index)	1.58

Table1. Characteristics of Ely marina water. Mean values May-October 1991.

The dry weight of the biofilm increases on both the biocidal and non-biocidal paints with increasing length of immersion but there is approximately 55% less on the equivalent biocidal paints (fig. 4). There is a reduction in dry weight with increasing depth on both biocidal and non-biocidal panels. Statistical analysis of the data shows that the dry weights from both treatments after 16w and 24w at depths 1 and 2 are significantly different to those at depths 4 and 5. Approximately 56% of the dry weight of the biofilm is accounted for as $CaCO_3$. Regression analyses show that organic weight and weight of $CaCO_3$ are linearly related with a regression R-Sq value of 80.2% (Heath *et al.*, submitted for publication).

All of the eight green algae and cyanobacteria isolated so far from calcified biofilms precipitate $CaCO_3$ in axenic culture. A typical growth precipitation curve for a unicellular green alga from the Chlorococcales is shown in fig. 3. The pH increases as the alga grows due to removal of CO_2 and HCO_3^- from the medium. At approximately pH 9.0, precipitation of $CaCO_3$ begins as evidenced by the rapid decrease in soluble calcium in the medium. The pH continues to rise up to 200h as CO_2 is removed by algal growth. Although growth continues at the same rate after 200h, the pH falls due to removal of CO_3^{2-} ions during the precipitation reaction. The large crystals of $CaCO_3$ associated with cells of the chlorococcalean alga at 200h can be seen in fig. 5.

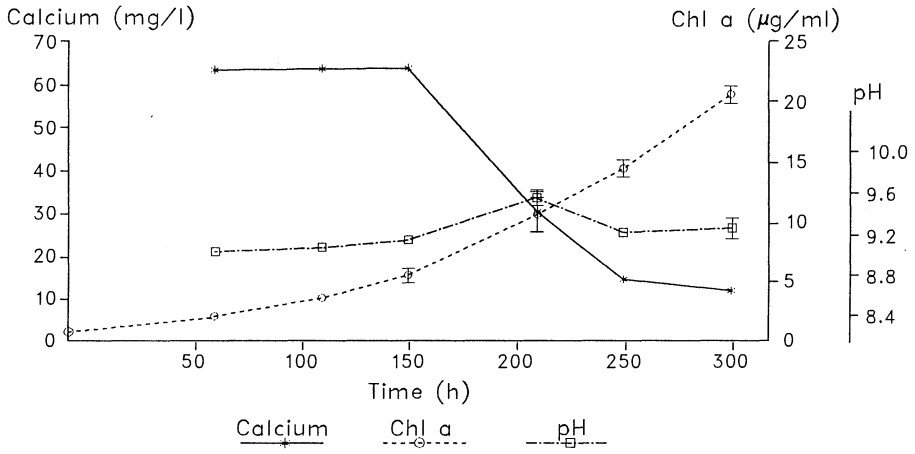


Fig. 3. Change in chlorophyll *a*, pH and soluble calcium during growth of a chlorococcalean alga. Each point is the mean of three determinations. Bars show S.E.

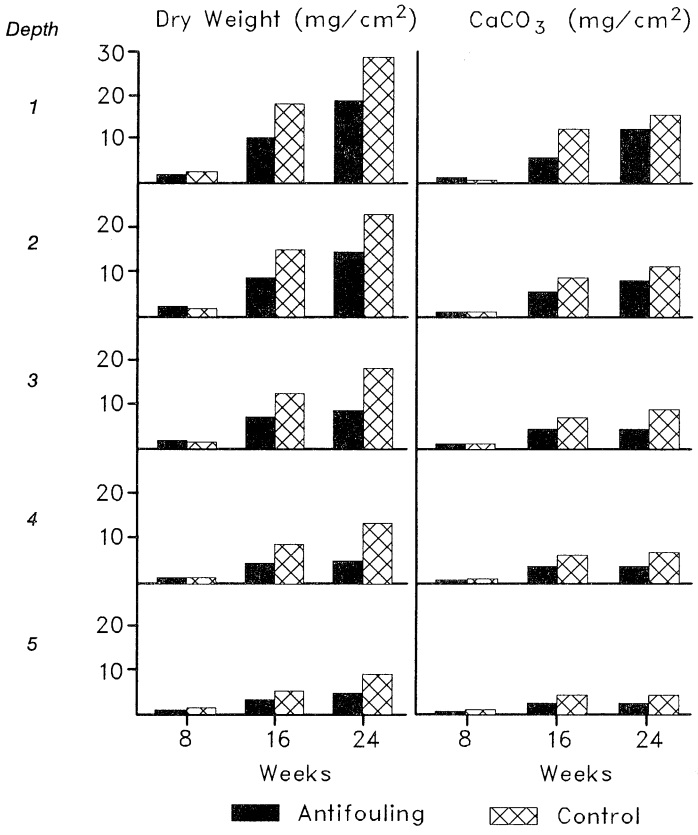


Fig. 4. Dry weight and weight of $CaCO_3$ of biofilms on non-biocidal (control) paint and antifouling paint sufaces after 8,16 and 24w immersion. Each value is the mean of four panels.

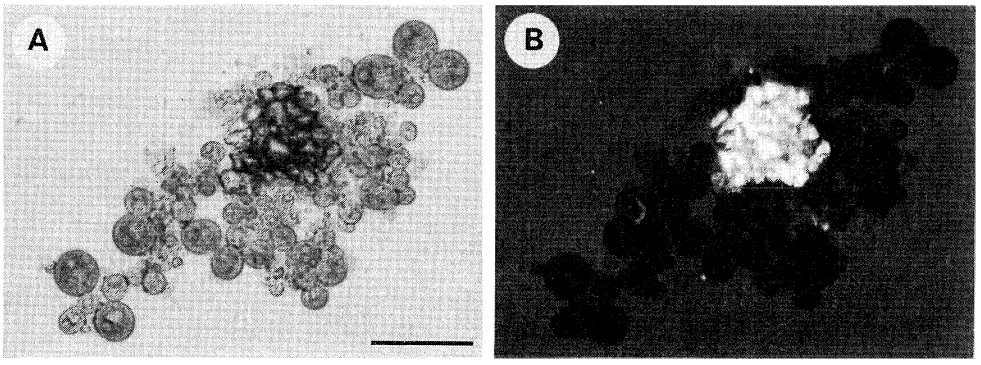


Fig. 5. Photographs of a group of cells of a green chlorococcalean alga after 200h growth. A = transmitted light, B= + polarizer showing calcite crystals. Scale bar = 50μm.

4. Discussion

The composition of water at Ely marina is typical of hard waters in which precipitation of $CaCO_3$ is seen (Effler, 1984). The SI did not vary with depth although the amount of $CaCO_3$ precipitated on panels did, thus it is unlikely that precipitation can be accounted for by chemical precipitation alone and it is postulated that the algal biofilm is involved in precipitation. Natural precipitation of $CaCO_3$ mediated by algae in Lake Constance has been described by Stabel (1986). Nucleation of calcite was initiated by phytoplankton during the summer when the saturation of the water increased.

Although there was only approximately 50% less weight of algal biofilm on the biocidal antifouling paint surfaces compared to the control surfaces, this is not unexpected since the water in the marina is static and water movement is necessary for optimum performance of antifouling paints. Weight of $CaCO_3$ was proportional to organic weight for both paints and at all depths and there was no evidence of species selection. It is possible that some species of alga are better able to precipitate $CaCO_3$ than others but all of the algae isolated precipitate in culture. Biofilm calcification observed here may therefore merely be the result of modification of the microenvironment (Simkiss, 1986) with the algae acting as nucleating surfaces and / or changing the microenvironment by physiological means. The laboratory data obtained so far would favour the latter.

5. Acknowledgements

Financial support from NERC and International Paint Ltd. is gratefully acknowledged.

6. References

Borowitzka, M.A. (1982) Mechanisms in algal calcification, *Progress in Phycological Research*, 1, 137-177.

Effler, S.W. (1984) Carbonate equilibria and the distribution of inorganic carbon in Saginaw Bay. *J. Great Lakes Res.*, 10, 3-14.

Evans, L.V. & Hoagland, K.D. (eds) (1986) *Algal Biofouling*. Elsevier, The Netherlands.

Grimshaw, H.M., Allen, J.A. & Parkinson, J.A. (1989) Nutrient Elements, Ch.5. In: Allen, S.E. (ed.) *Chemical Analysis of Ecological Materials*. Blackwell Scientific Publications.

House, W.A. (1984) The kinetics of calcite precipitation and related processes. *FBA Annual Report*, No. 52. pp.75-90.

Kelts, K. & Hsu, K.J. (1978) Freshwater carbonate sedimentation. In: *Lakes: Chemistry, Geology, Physics*. Lerman, A. (ed). Springer, pp.295-323.

Jeffery, S.W. & Humphrey, G.F. (1975) New spectrophotometric equations for determining chlorophylls a,b,c_1 and c_2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanzen.*, 167 S, 191-194.

Makereth, F.J.H., Heron, J. & Talling, J.F. (1978) *Water Analysis: Some Revised Methods for Limnologists*. Freshwater Biological Association, Scientific Publication No. 36. pp. 50-53.

Simkiss, K. (1986) The Processes of Biomineralization in Lower Plants and Animals - An Overview. In: Leadbeater B.S.C & Riding R. (eds) *Biomineralization in Lower Plants*. Clarendon Press, Oxford. pp 19-37.

Stabel, H.H. (1986) Calcite precipitation in Lake Constance: chemical equilibrium, sedimentation and nucleation by algae. *Limnol. & Oceanogr.* 31, 1081-1093.

CONTROL OF STAPHYLOCOCCAL ADHESION TO MODEL BIOPOLYMERS

G.W. HANLON
S.P. DENYER
Department of Pharmacy, Brighton Polytechnic, Brighton BN2 4GJ, U.K.

J. BRIDGETT
M.C. DAVIES
Department of Pharmaceutical Sciences, University of Nottingham, Nottingham U.K.

1. Introduction

Indwelling medical devices and implants such as sutures, catheters, prosthetic heart valves and artificial joints are increasingly used in modern medicine. This wide variety of biomaterial components has been created to meet the specific functional requirements of systems and organs. The materials from which they are manufactured can be polymers, metals, ceramics and sometimes modified natural materials. There is, however, an increasing realisation that these existing materials have their limitations and further developments will require a more thorough understanding of the mechanisms underlying current problems (Jansen et al 1988).

Infection is a frequent complication associated with indwelling biomaterials and can occur in both transient and permanent devices usually via the formation of a bacterial biofilm. This complication covers a wide spectrum of products, including heart valves, pacemakers, cerebrospinal fluid shunts, vascular, peritoneal and urinary tract catheters, IUCDs and artificial joints. At best, infective episodes may mean failure of the device, at worst a fatal intractable infection may develop A mortality rate of up to 60% has been reported (Stamm, 1978). Control of these infections has been sought through classical antimicrobial measures but an established bacterial biofilm is usually resistant to antimicrobial therapy and often the only successful approach involves removal of the device (Costerton, 1984; Gristina and Costerton, 1984).

The extent of bacterial adhesion to surfaces has frequently been related to substratum characteristics such as surface free energy, critical surface tension, work of adhesion and surface charge. While the results from such studies are frequently contradictory, probably reflecting experimental variation and macromolecular conditioning of biomaterial surfaces, there is a body of evidence to suggest that hydrophilic surfaces generally exercise the best control over microbial adhesion. As a consequence, attempts have been made to understand the nature of the adhesive events involved in bacterial biofilm formation and to seek to control these by biomaterial design. However, this avenue demands a clear appreciation of the importance of surface properties of both biomaterials and microorganisms in the resultant interactions, and must also pay attention to the influence of environmental factors on both of these surfaces.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 557–565.

There are two main approaches to the design of new biocompatible polymeric materials. An entirely novel polymer with the appropriate mechanical and surface properties may be manufactured or alternatively, the surface of existing, mechanically suitable, materials may be modified to give the required characteristics.This paper describes studies in which the surfaces of different biomaterials were modified, by various means, to increase their hydrophilic nature. The influence of these changes on the adhesive properties of selected microorganisms is described.

2. Experimental

Seven clinical isolates of *Staphylococcus epidermidis* were obtained from patients suffering from peritonitis as a complication of continuous ambulatory peritoneal dialysis (CAPD). The surface characteristics of the isolates were studied using hydrophobic interaction assay (HIA), contact angle measurements, zeta potential and electrophoretic mobility. The data are presented in Table 1 and illustrate that groups of isolates can be distinguished based on their relative hydrophobicities with strain 901 being most hydrophobic, strain 900 the least hydrophobic and strain 904 intermediate between the two. The remaining strains were indistinguishable from strain 904. The data for electrophoretic mobility and zeta potential do not readily allow any distinction to be drawn between the different strains nor any relationship with hydrophobicity to be determined.

Table 1. Surface characteristics of clinical isolates of *S. epidermidis* HIA ranking - 1=most hydrophobic, 3=least hydrophobic. All figures ±SD.

STRAIN	Θ_W	ZETA POTENTIAL (mV)	ELECTROPHORETIC MOBILITY ($\mu MV^{-1}s^{-1}$)	HIA
900	15±2	-41.5±0.2	-3.18±0.02	3
901	38±2	-40.0±0.7	-3.05±0.03	1
902	25±2	-43.2±0.4	-3.40±0.03	2
903	25±2	-43.6±0.2	-3.33±0.02	2
904	26±2	-44.6±1.0	-3.44±0.05	2
905	23±2	-48.7±2.0	-3.74±0.10	2
906	26±2	-44.2±0.3	-3.38±0.02	2

Bacterial adhesion studies were carried out using washed cell suspensions at a density of $5x10^8$ cells per ml. Surfaces were exposed to the suspensions for up to 4 hours at 37°C with intermittent gentle shaking and then removed, washed with Phosphate Buffered Saline (PBS) and examined for adherent bacteria. Bacterial adhesion was assessed initially by cellular ATP extraction and firefly bioluminescence measurement and subsequently by epifluorescence microscopy and image analysis.

2.1. OXYGEN GLOW DISCHARGE SURFACE TREATMENT

Polystyrene was used as the base material for initial adhesion studies and was either spun or dip cast as a thin film onto glass from a 5%w/v toluene solution. The surface of the polystyrene was then rendered hydrophilic to varying extents by oxygen glow-discharge treatment (Polaron, Watford) to yield surfaces with water contact angles between 0 and 90 degrees. The treated polymer surfaces were analysed using advancing water contact angle determinations and X-ray photoelectron spectroscopy (XPS) . The characteristics of the modified surfaces are given in Table 2. XPS demonstrates an increasing incorporation of oxygen into the surface with extended plasma treatment probably leading to the progressive generation of hydroxyl, carbonyl, carboxyl and carbonate moieties. Contact angle determinations show an increase in hydrophilicity with increasing time of treatment until at 10 seconds the surface is completely water wettable. Adhesion studies were undertaken using the representative strains 900, 901 and 904 according to the general methods described earlier.

Table 2 Surface characteristics of oxygen plasma treated polystyrene.

TREATMENT TIME (s)	θ_W	γ_c (mJM^{-2})	ATOMIC % OXYGEN
0	90	33.9±2.1	8
0.1	65	40.0±3.0	10
0.5	45	40.0±4.0	13
1.0	30	41.5±5.0	18
3.0	20	_	19
10.0	0	_	23

Using the indirect method of ATP bioluminescence it could be shown that all strains of bacteria exhibited a reduction in adhesion when the polystyrene surface was modified to give a zero water contact angle. Further examination of bacterial adhesion by epifluoresence microcopy revealed that the bacteria do not deposit evenly over the surface but adhere in clusters suggesting a combination of cell-cell attraction and cell-substratum attraction. The cells initially adhere in small clusters which increase in size with time and eventually coalesce to form a reticulated pattern of adhesion.

When the adhesion of the three strains 900, 901 and 904 was subjected to detailed image analysis, adhesion was found to be greatest at water contact angles of 30°, 65° and 45° respectively (Figure 1). On surfaces showing this "window of maximal adhesion" the cluster sizes do not increase as dramatically as they do on other surfaces (see Figure 2), suggesting that on these surfaces the cell-substratum interactions are more substantive and dominate over the cell-cell interactions. The presence of three different windows of maximal adhesion for the strains examined illustrates the importance of bacterial surface characteristics in bioadhesion studies of this type. It also indicates that hydrophobic interactions may not be the sole mediator of adhesion, since a surface with a water contact angle of 90 is not maximally adherogenic. Nevertheless, adhesion significantly decreased from the peak uptake at the window of adhesion as the surface was made more hydrophilic implying some contribution from hydrophobic interactions.

2.2. PLURONIC SURFACTANTS

A further approach to surface modification was to investigate the effect of sterically stabilised biomaterials using pluronic surfactants.These have been shown to minimise protein adhesion and present themselves as possible candidates for the control of microbial adhesion . Polystyrene surfaces were treated with 2%w/v pluronic block co-polymer surfactants for 20 hours at 25°C. After treatment the surfaces were rinsed thoroughly in distilled water. Adhesion studies were carried out using *S. epidermidis* strain 900 as already described and analysed by microscopy as before.

Figure 3 shows the effect of pluronic treatment on the adhesion of strain 900. Clearly, all surface treatments significantly reduce bacterial adhesion levels over untreated polystyrene controls with the exception of L31. It is assumed that a sterically stabilised surface is created by pluronic adsorption via the hydrophobic PPO chain leaving the hydrophilic PEO chain protruding from the surface . In this way the protruding PEO chains create a barrier to the approach and initial attachment of bacteria. It has been suggested that the chain lengths of both the anchoring PPO block and the protruding PEO blocks will influence the effectiveness of the sterically stabilised layer in reducing adhesion. The precise nature and balance of the PPO/PEO chains necessary to minimise adhesion is unclear although the poor performance of L31 may be explained as being the result of the combination of the short PPO and PEO chains within the molecule. The use of pluronic surfactants for the coating of medical devices may have important potential but, as yet, has not been assessed clinically.

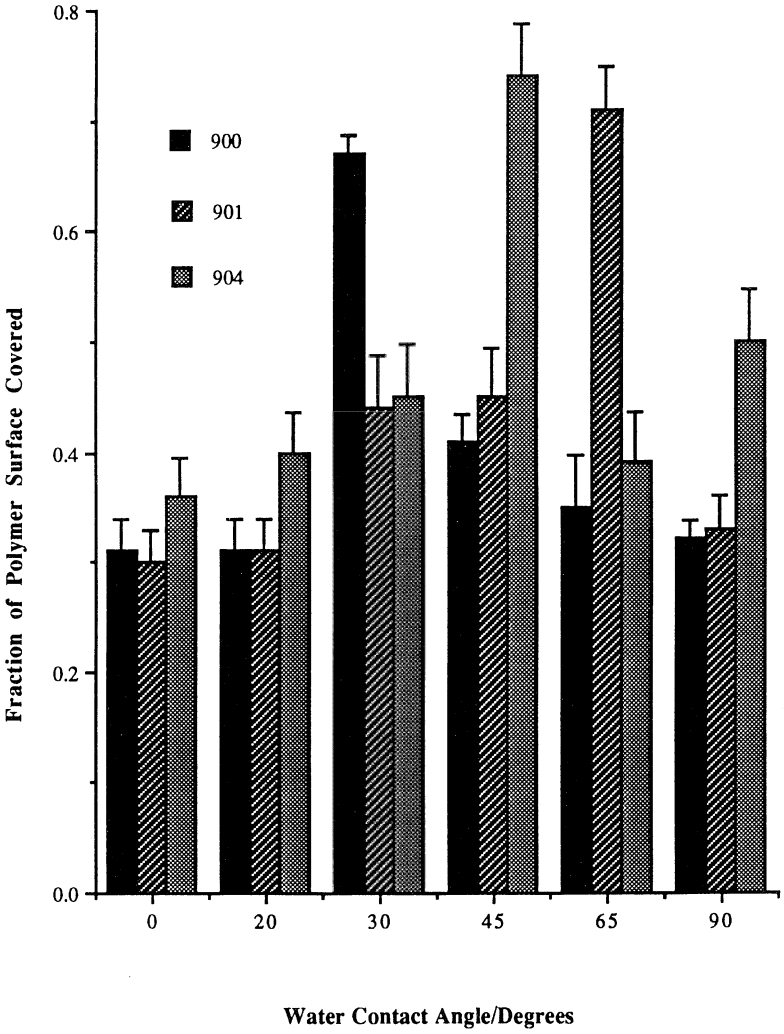


Figure 1. Adhesion after 4 hours contact of three strains of *S. epidermidis* to oxygen plasma treated polystyrene having a range of water contact angles.

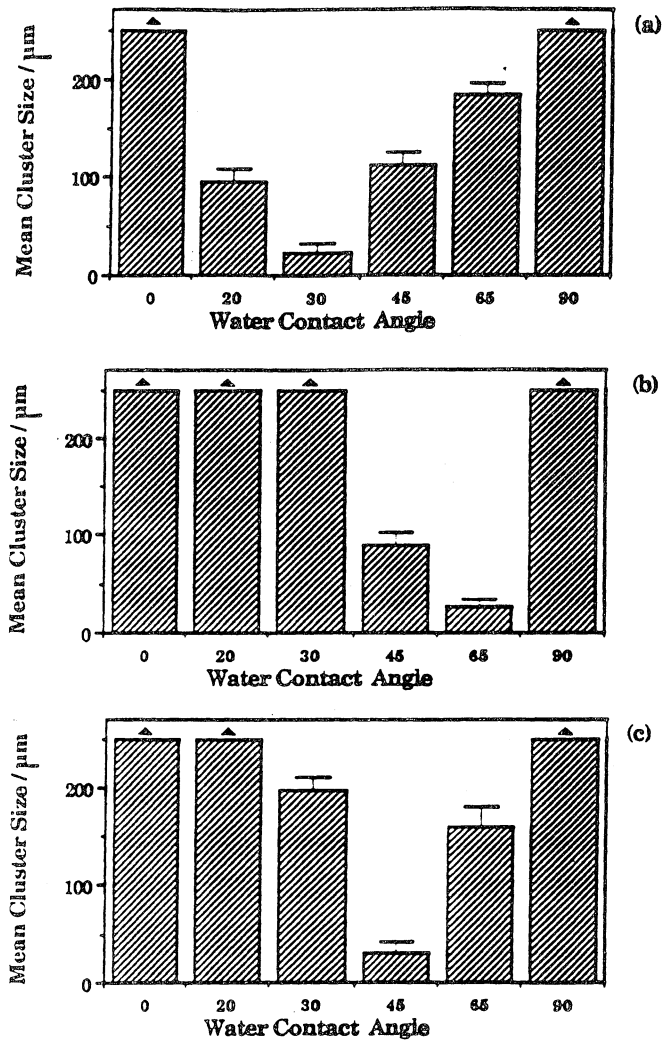


Figure 2. Mean cluster size of adherent *S. epidermidis* as a function of substratum water contact angle. (a) strain 900, (b) strain 901 and (c) strain 904. Mean ±S.D. shown, n=3.

2.3. HYDROGEL COATING

Hydrocephalus is managed surgically by the drainage of accumulated cerebrospinal fluid (CSF) from the cerebral ventricles via ventriculo-peritoneal (VP) shunts (James et al 1980). VP shunts are usually made from medical grade silicone rubber and infection rates for shunt procedures of up to 35% have been reported (Bayston et al, 1983). The majority of these infections occur within the lumen of the shunt and arise within 3 months leading to a blocked catheter resulting in abdominal pain and raised intracranial pressure (Price, 1984). As indicated previously the only really successful approach to the management of these infections involves removal of the device and implantation of a new shunt only after antimicrobial therapy has cleared the infection. In common with many catheter associated infections the main infecting agent has been shown to be *S. epidermidis*. Other skin bacteria such as *S. aureus*, diphtheroids and occasional Gram negative bacteria have also been isolated with lower frequency (Price, 1984).

The impetus to modify catheter surfaces has arisen not in an attempt to reduce infection but because of a requirement to minimise trauma to the tissue upon insertion and also to improve tissue compatibility. Many workers have reported modification of catheters using hydrogel coatings which can swell in water and retain a significant fraction of water within their structure but not dissolve.

In order to study bacterial attachment to hydrogel coated devices VP shunts were used in the first instance which were both untreated and also Hydromer coated (a hydrogel prepared by the interaction of PVP with one of several isocyanate prepolymers). These were obtained from Mediplus Ltd. (Buckinghamshire U.K.). 8mm lengths of shunt tubing were mounted on glass microscope slides. The sections were cut lengthways and the cut edges were pulled apart using metal plates, thus exposing the inner surface of the shunt tubing. The opened tubing was held flat on the microscope slide and secured using metal clips. Hydromer coated and uncoated silicone rubber VP shunts were then placed in PBS at 37°C for one hour before being exposed to washed bacterial suspensions as described before. The bacteria used were *S. epidermidis* strains 900, 901 and 904 together with 2 further strains of *S. epidermidis* and one strain of *S. aureus* isolated from CSF shunt infections. After treatment the loosely adherent bacteria were removed in a stream of PBS and examined using epifluorescence image analysis.

The results presented in Figure 4 clearly illustrate that the Hydromer coating reduces bacterial adhesion levels by 84-92% over the controls. It is assumed that the hydrophilic nature of the Hydromer coating renders the shunt surface energetically unfavourable with respect to attachment and hinders the establishment of short range hydrophobic interactions between cell and biomaterial surface. Nevertheless, adhesion does still take place onto the Hydromer coated device and the clinical implications of this may be only to delay any potential onset of infection.

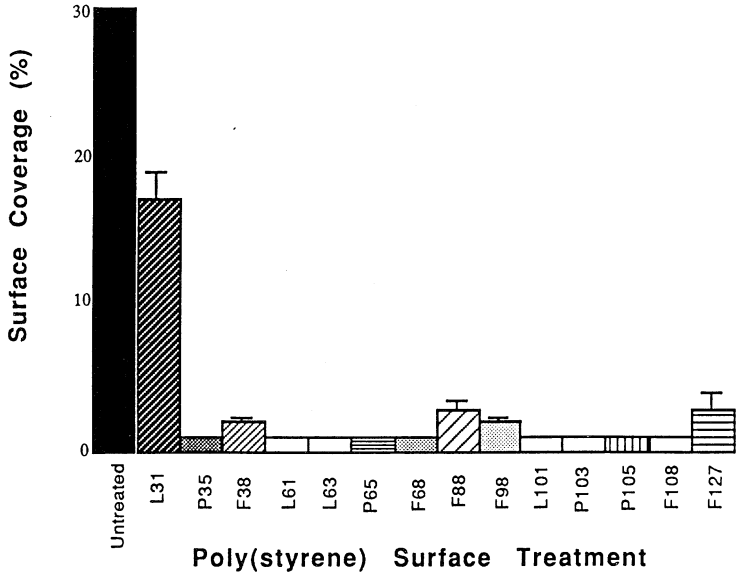


Figure 3. Effect of polystyrene surface treatment with a range of pluronic surfactants on adhesion of *S. epidermidis* (strain 900).

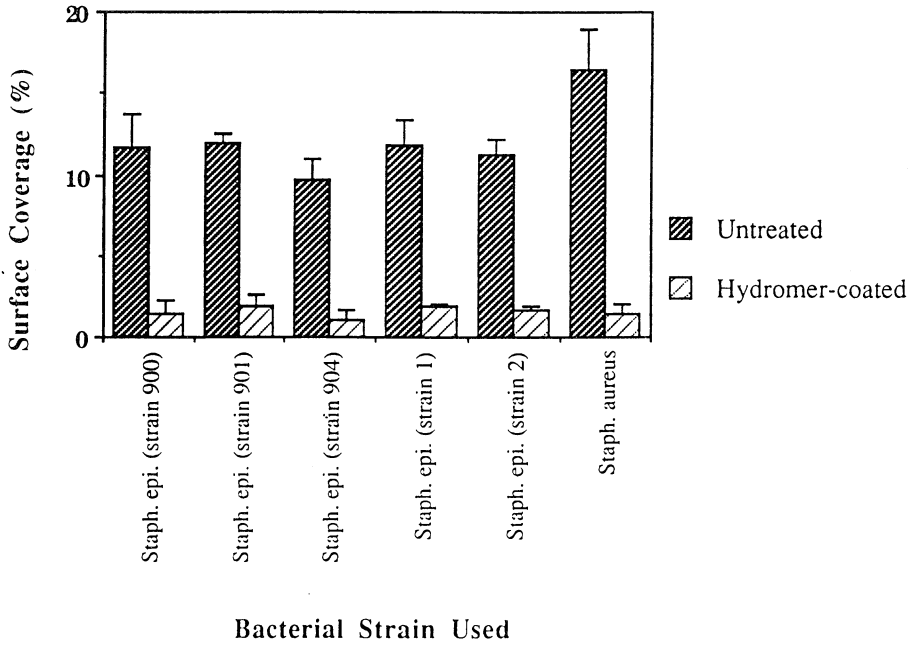


Figure 4. Adhesion of different bacteria to untreated and Hydromer-coated VP shunts.

3. Conclusions

Despite the availability of many antimicrobial drugs infection remains one of the major complications of the use of indwelling medical devices. Approximately 45% of hospital acquired infections are associated with such systems (Stamm 1978). Bacterial adhesion to the implant is the initial stage in the pathogenesis of device-related infections and is a multifactorial process, dependent upon the surface properties of both the bacterium and the substrate. Surface modifications to biopolymers can lead to a significant retardation in colonisation level with obvious advantages to the host where successful defence may rely on an early and effective response against the microbial challenge.

Surface coating with hydrophilic polymers may achieve some degree of success through a combination of the hydrophilic nature of the coating and other factors such as reduced microrugosity or increased surface mobility.

References

Bayston R, Leung T.S.M., Wilkins B.M. and Hodges B. (1983) Bacteriological examination of removed cerebrospinal fluid shunts. J.Clin. Path. **36** 987-990.

Costerton J.W. (1984) The etiology and persistence of cryptic bacterial infections: a hypothesis. Rev. Inf. Dis. **6** (Suppl. 3) S608.

Gristina A.G. and Costerton J.W. (1984) Bacterial adherence and the glycocalyx and their role in musculoskeletal infections. Orthop. Clin. North Am. **15** 517-535.

James H.E., Walsh J.W. and Wilson H.D. (1980) Prospective randomized study of therapy in cerebrospinal fluid shunt infections. Neurosurgery **7** 459-463.

Jansen B., Peters G. and Pulverer G. (1988) Mechanisms and clinical relevance of bacterial adhesion to polymers. J. Biomat. Appl. **2** (4) 520-543.

Price E.H. (1984) *Staphylococcus epidermidis* infections of cerebrospinal fluid shunts. J. Hosp. Inf. **5** 7-17.

Stamm W.E. (1978) Infections related to medical devices. Ann. Int. Med. **89** 764-769.

THE USE OF BIOCIDES IN INDUSTRY

T.R. BOTT
School of Chemical Engineering
University of Birmingham
Edgbaston
Birmingham B15 2TT. UK

1. Introduction

The problems of biofouling may be manifest in a number of industries, such as food processing, bioreactors and associated equipment but by far the greatest problems are encountered in cooling water systems and in drinking water treatment. Unless action is taken to reduce or eliminate the extent of biofilms costly operational difficulties are likely to occur. The obvious way to tackle the problem is by the use of biocides to kill the micro-organisms that constitute the biofilm. The application of biocides however, has to be considered carefully if other problems, such as environmental pollution and of course high costs, are to be avoided. Although the use of biocides can still in some ways, be regarded as "an art", over the past few years there have been considerable advances in their application.

It will be readily appreciated that biofilms will be much more resistant to a biocide than planktonic micro-organisms. Even though all suspended micro-organisms may be killed by the biocide, the cells constituting the biofilm may well be still active. The biofilm and associated extracellular polymers represents a resistance to penetration by the biocide. Unless the biocide is able to diffuse to the lower layers of the biofilm only the micro-organisms near the surface will be affected. Furthermore there is a problem of mass transfer to the biofilm surface itself, through the bulk liquid. In static systems where there is little bulk movement, mass transfer to the surface will be largely dependent upon molecular diffusion or Brownian motion. If, on the other hand, the aqueous suspension of micro-organisms is flowing under turbulent conditions across the surface on which the biofilm is formed, mass transfer to the surface will be enhanced by eddy diffusion. Not only does this improve the transport of biocide present in the flowing liquid towards the surface, it will of course also enhance the movement of micro-organisms and microbial nutrients to the surface. Even under turbulent conditions there will be a resistance to mass transfer through the bulk liquid to the active surface, in addition to the resistance to diffusion within the biofilm.

Although the true turbulence criterion is the Reynolds number, velocity may be taken as a rough indicator for flow inside tubes of a

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 567–581.

size usually found in shell and tube heat exchangers. It has been demonstrated how the effectiveness of hypochlorite solution (a source of hypochlorous acid) is affected by velocity (Nesaratnam and Bott 1984). High velocities (say >1 m/s) favour removal as do relatively high concentrations of "free" chlorine (say > 4 mg/ℓ).

Crevices and "hideaways" for micro-organisms may not be adequately penetrated by biocides and these pockets of activity represent an inoculum for rapid biofilm growth if the biocide treatment is not maintained on a continuous basis.

Unless the biofilm is removed from the surface say by the combined action of a biocide and fluid shear at the surface, the problems associated with the biofilm remain. On the other hand if "shock treatment" is employed and the biofilm even though dead, comes away from the surface in large pieces further problems may result. If the dislodged pieces of biofilm are sufficiently large for instance, blockage of flow channels and pipes can occur downstream. Blockage on the tube plates of a shell and tube heat exchanger is a particular problem that may arise from this cause. The only remedy may be to shut down operations and clean the equipment. The situation that the use of the biocide was deemed to avoid!

Moreover where the biocide that is applied reacts chemically with organic matter (for instance chlorine), the presence of large quantities of dead biological material in suspension can require relatively large concentrations of biocide to be effective against active micro-organisms. In addition the breakdown products of these chemical reactions may provide nutrients for accelerated micro-organism growth on the surfaces of equipment in contact with the suspension.

The discussion of the use of biocides will be made largely in respect of cooling water systems, with reference where appropriate, to other industrial applications.

2. The Ideal Biocide and Aspects of Choice

In cooling water systems a wide range of micro-organisms is likely to be encountered, with its own ecology that will be very dependent upon the particular site. At some stage, the water (say from a cooling water system) is discharged either to a natural water system, eg. a river or lake, or to the drainage system, the use of a persistent (and possibly harmful) biocide is unlikely to be acceptable because of the risk for the environment. Cooling water usually requires other treatment in addition to the use of biocides to prevent corrosion of equipment, or the deposition of scale-forming salts. Clearly chemical reactions between the various additives is unacceptable since this will reduce the overall efficiency of the total treatment programme.

These and other requirements for an ideal biocide may be summarised:

1. Active against a wide range of micro-organisms.
2. Relatively low toxicity to other life forms.
3. Biodegradable, ie. the necessary residual biocide in the water is rendered harmless by further biological activity after a time lapse.
4. Non-corrosive.

5. Effectiveness not impaired by the presence of inorganic or organic materials other than micro-organisms present in the system.
6. Does not deactivate other additives, eg. corrosion inhibitors or scale preventatives used in the general treatment programme.

It is extremely unlikely that all biocides will meet all these criteria so that the final choice of a particular biocide will be a compromise. Not least in the consideration will be the cost of a particular treatment.

Factors other than cost will also have to be taken into account in the choice of a biocide for a specific site application and include:

1. Quality of the water (make up or once through systems).
2. The extent and nature of the biological contamination.
3. Contact with the atmospheric environment (largely cooling water recirculating systems using cooling towers or spray ponds).
4. Process leaks into the system which may:
   a) provide nutrients for biological growth
   b) act as a biocide
5. Residence time in the system in relation to "half life".

Of these factors the most important are likely to be those associated with the quality of the water and the microbial contamination. The chemical composition of the water and the pH may have a pronounced effect on the efficacy of a particular biocide. For instance it may be necessary to operate at a particular pH to reduce the potential for precipitation of hardness salts. There may only be a limited number of biocides that will be effective at this pH.

A review of cooling water management (Bott 1988) and biofouling in water treatment (Flemming 1991) give some insights into the requirements of biocidal treatment.

3. Classification of Biocides

Biocides may be conveniently classified according to their chemical character, ie. either oxidising or non-oxidising. Tables 1 and 2 list some of the more common biocides under each of these headings.

These tables are not, by any means, exhaustive, but they do illustrate the range of additives available. Other chemicals display biocidal qualities and new products are continually being developed to meet the stricter and necessary, controls that are being imposed for environmental protection.

4. Some Biocides in More Detail

It is outside the scope of this discussion to consider all the biocides that are available. It is useful however, to outline the qualities of some of the more common additives since this will illustrate aspects of their use in industrial plant.

4.1 CHLORINE

Chlorine is by far the most widely used biocide particularly in cooling

TABLE 1. Oxidising biocides

Biocide	Availability
Chlorine	Liquid (compressed gas) Sodium hypochlorite solutions Calcium hypochlorite powder Organic compounds - chlorine donors *In situ* generation, eg. electrolysis of brine
Chlorine dioxide	Usually generated on site by reaction between sodium chlorite and chlorine
Bromine	Liquid Hydrolysis of compounds like halogenated hydantoins Displacement from a bromine salt water by chlorine
Ozone	Generated on site by electrical discharge in dry air or oxygen followed by absorption in water

TABLE 2. Non-Oxidising Biocides

Biocide	Availability
Chlorinated phenols	Usually sodium salts of trichlorophenol and pentachlorophenol
Copper salts, eg. copper sulphate	As solution
Organic and inorganic compounds containing Mercury	As solution
Amines and ammonium compounds	Straight chains or quarternary compounds
Organo tin	Liquid
Organo sulphur compounds	Liquid complex organic molecules containing sulphur
Isothiazolones	Liquids
Acrolein	Compressed gas
Organo-dibrominated compounds	Liquid
Thiocynates	As a solution

water systems where large volumes of water are used. It has a wide spectrum of activity and is effective against macro- in addition to micro-organisms. It will be discussed in some detail since its established use highlights the opportunities and problems associated with the use of biocides.

Chlorine gas reacts with water and is hydrolysed to form two acids, hypochlorous and hydrochloric acid, respectively.

$$Cl_2 + H_2O \rightarrow HOCl + HCl$$

Hypochlorous acid will ionise according to this reversible reaction:

$$HOCl \rightleftharpoons H^+ + OCl^-$$

The amount of hypochlorous acid, as opposed to hypochlorite ion, determines the biocidal efficacy.

The pH of the cooling water is directly responsible for the extent of ionisation of hypochlorous acid (Marshall and Bott 1988). At pH 5.0, there is very little ionisation. At pH 7.5 there are approximately equal amounts of acid and hypochlorite ion. Chlorine becomes ineffective as a microbiocide at pH 9.5 or greater, as a result of total ionisation. A pH range of 6.5 - 7.0 is considered a practical range for chlorine-based microbial control programmes, since lower pH values would increase system corrosion.

Hypochlorous acid is an extremely powerful oxidising agent. It easily diffuses through the cell walls of micro-organisms, and reacts with the cytoplasm to produce chemically stable nitrogen-chlorine bonds with the cell proteins. Chlorine oxidises the active sites on certain co-enzyme sulphydryl groups which constitute intermediate steps in the production of adenosine triphosphate, which is essential to respiration. Hypochlorous acid is estimated to be twenty times more reactive (effective) as a microbiocide than the hypochlorite ion.

Chlorine is widely used as a biocide largely on account of:

1. Its relatively low cost.
2. Its effectiveness; usually the required residual concentration is of the order of 0.5 - 1.0 mg/ℓ .

When chlorine is added to a system it will react with different materials contained in the system, eg. organic materials or inorganic compounds such as ammonia, ferrous ion and sulphate. Under these circumstances chlorine is consumed and is no longer effective as a biocide. For this reason sufficient chlorine must be added to satisfy the chlorine demand (for reactions), and leave a residual which will perform the biocidal function. If large quantities of oxidisable material are present in the system large quantities of chlorine may be required for it to be effective.

Continuous chlorination is clearly the method of control for good results but it may not be economically feasible.

In recirculating systems employing a cooling tower or spray pond, chlorine will be lost from solution into the atmosphere. In once through systems using large volumes of water, continuous dosing with chlorine could result in very high costs. Additions of chlorine on an intermittent basis may be necessary for such systems. The free residual requirement and the dosing frequency will largely be a matter for trial

and error in relation to the site factors. As a guide, intermittent treatment of 2 or 3 hours per day with free residual concentrations of up to 1 mg/ℓ may be found satisfactory. Unless there is some automatic system of dosing, manual dosing provides the opportunity for omission with consequent ineffective operation.

Some comments on chlorine treatment programmes are contained in Table 3 (Betz Laboratories Inc. 1976).

Low levels of residual chlorine may be regarded as a biostat, ie. preventing colonisation or restricting growth. In a particular laboratory test (Kaur et al 1988) chlorine levels as low as 0.2 - 0.6 mg/ℓ inhibited biofilm attachment and growth. The effectiveness of chlorine administered as hypochlorite solution for biofilm removal was illustrated in some laboratory experiments (Nesaratnam and Bott 1984). At a velocity of 1.2 m/s (Reynolds number = 17460) with a continuous dose of free chlorine at a concentration of 1 mg/ℓ 40% of a particular biofilm was removed, whereas under similar conditions with 4 mg/ℓ over 80% of the biofilm had been removed.

TABLE 3. Chlorine dosing programmes

Programme	Remarks
Continuous chlorination - free residual	Most effective Most costly Not always technically or economically feasible due to high chlorine demand
Continuous chlorination - combined residual	Less effective Less costly Inadequate for severe problems
Intermittent chlorination - free residual	Often effective Less costly than continuous chlorination
Intermittent chlorination - combined residual	Less effective Least costly

Although a chlorine residual is present in the water fed to a cooling tower this does not necessarily mean that a residual will be seen in the basin of the tower. As already described, oxidisable material will reside on the tower internals and having a chlorine demand will reduce the free chlorine in the water. In addition the stripping effect of the air passing through the tower will further reduce the chlorine level.

Because of the increasing environmental concern for chlorine discharge the technique of targeted chlorine addition represents an alternative to current practice (EPRI 1985). The method also allows the use of relatively high chlorine doses for short periods, which are known to be more effective than low concentrations for longer times. In targeted chlorine dosing a fraction of the heat exchanger tubes (eg. a section of

a condenser on a power plant) are exposed to the biocide at any one time, thereby permitting higher chlorine concentrations in the bulk water within these relatively few tubes. For example it is possible to feed 10% of the tubes at any one time, using 10 times the permissible allowable chlorine concentration since when the water is recombined at the outlet the final concentration will be reduced by a factor of 10 compared to the dose in the target tubes.

The penalty for the benefits of this technique is the higher cost of the more complex dosing equipment required compared to the more usual blanket chlorination techniques. The targeted dosing technology lends itself to automatic control with little or no operator involvement. Optimum cycle times are necessary based on local water quality, to ensure the maximum advantages from the method.

Future developments may involve targeted chlorine injection into the viscous sub-layer region adjacent to the surfaces being contaminated, to reduce the chlorine discharge still further.

Some operators of cooling water plant chlorinate and add a non-oxidising biocide on a low frequency basis, to achieve a more effective microbiological control.

Other aspects of the use of chlorine as a biocide which need to be kept in mind are:

1. If the pH of the circulating water is allowed to rise for operational reasons, eg. less frequent blowdown (to reduce water costs) resulting in greater concentrations of alkaline salts, the effectiveness of chlorine as a biocide decreases rapidly above a pH of about 7.5 when chlorine (liquid or gas) is used. Hypochlorite ions are effective at higher pH.
2. For thick slimes the penetration of chlorine may not be effective in restricting growth. Under these circumstances sulphate reducing bacteria (SRB) can become a problem if thick slimes have been allowed to develop. It has been shown however, that the penetration (Nesaratnam and Bott 1984) and removal (APV 1982) of slime layers is very dependent upon the water velocity across the surface of the biofouling.
3. Since chlorine in water produces acid conditions when chlorine (liquid or gas) is employed, excessive use of chlorine treatment can result in corrosion of metal surfaces. This is not the case when hypochlorite is used as the source of chlorine. Wooden internals of cooling towers can also be affected by the use of chlorine since it has a tendency to delignify the wood, but this is generally not a problem because continuous chlorine concentrations of 5 - 10 mg/ℓ or more are required for this to occur.
4. Because of the potential hazardous and environmental problems associated with the use of chlorine for the treatment of cooling water, particularly for large power plant installations, the transport, storage and handling of large quantities of liquid chlorine should not now be considered. If chlorine is to continue as a biocide it will be necessary to use the more expensive methods of on site generation, eg. by electrolysis or the use of hypochlorite solutions. The continued use of chlorine however, is likely to be in doubt (see 5 below).

5. Reactions of chlorine with organic materials mentioned earlier, produce chloro-organic compounds that are considered to be toxic and carcinogenic. Furthermore chlorine and chlorine compounds persist in the environment and are likely to build up over a period of time, with the distinct possibility that chlorine will enter the food chain. Because of environmental legislation associated with chlorine residuals in water discharge, it may be necessary to consider some method of dechlorination by the addition of common reducing agents (eg. sulphur dioxide, sodium thiosulphate, sodium sulphate). The additional costs involved, however, may make chlorine less acceptable than other biocides with a limited life (eg. ozone).

4.2 OZONE

Ozone has been used for drinking water purification for a number of years (Rice et al 1981). Its potential use as a biocide for industrial cooling water applications has not been fully explored although development work is well advanced.

Ozonised air (or oxygen) is usually produced on site by electrical discharge using dry air (or oxygen) and the ozone is dissolved in the water to be treated in suitable equipment (traditionally a bubble column). Other techniques for the production of ozone are being developed with the objective of reducing the relatively high cost of the standard discharge method.

In the water industry ozone is becoming a preferred pre-treatment disinfectant to overcome the problems of chlorine reactions with organic materials mentioned earlier. For cooling water applications for biocidal treatment and the removal of biofilms it is envisaged that a "biocide solution" would be made by dissolving ozone in water to a relatively high concentration and injecting this ozonised water into the bulk cooling water to produce the desired treatment concentration.

In many respects ozone contained in water is a useful biocide since the life of ozone is relatively short, which reduces its potential environmental and safety problems. There are no problems associated with storage, because of its short life ozone has to be generated in response to demand. At the same time this produces potential difficulties since there can be no "buffer storage" in the system.

The application of ozonised water as a biocide will be similar to the use of chlorine. In particular it will be necessary to ensure that a residue of active ozone is left in solution. The oxidised products from the treatment will be quite different from those produced by chlorine. Bearing in mind that site conditions are specific, tests carried out at a particular power station (EPRI 1980) revealed that although ozone was not as effective as chlorine on a weight basis, it can be successfully employed at low level (0.6 mg/ℓ) and was successful in the prevention of biofouling. Based on considerations of time-concentration treatment combinations in fresh water systems however, ozone is superior to chlorine (Garey 1979). Laboratory tests (Kaur et al 1988) confirmed that concentrations as low as 0.1 mg/ℓ ozone were effective in the removal of biofilms (thickness ~ 150 μm) from test surfaces.

Based on the literature and experience Rice and Wilkes (1991a) have

given a list of the claimed benefits of ozone in cooling water applications:

1. Many claims are made (with significant supporting data) that ozone can be the sole chemical treatment.
2. In many cases minimal or zero bleed-off is required in systems treated only with ozone.
3. Ozone is claimed to provide not only control of biofouling organisms, but also control of scaling and corrosion.
4. In ozone-treated systems, significant quantities of calcium, magnesium, silica, alkalinity and TDS are reported to be removed from recirculating cooling water.
5. Ozone decomposes to oxygen as an end product and thus does not introduce environmentally unacceptable substances into the cooling water.
6. When ozone is applied as a standalone treatment of cooling water, significant benefits in pollution control, water conservation (due to reduced blowdown), and treatment cost reduction have been demonstrated.

One of the major problems concerning the introduction of ozone as a biocide is an inadequate understanding of the chemistry of ozone in relation to the constituents of the water at a particular site. Points in respect of ozone chemistry have been made by Rice and Wilkes (1991b). The important comments are:

1. Ozone decomposes rapidly when added to water, as compared with other biocidal oxidants used in water treatment. In very pure waters, this decomposition rate is slow, and the half-life of ozone is on the order of hours. However, in "real-world" recycling cooling tower waters, which contain relatively large quantities of ozone-demanding impurities, the half-life of ozone is measured in terms of minutes, sometimes in the 1 to 10 minute range, after the immediate ozone demand of the water has been satisfied. With very "dirty" waters, the half-life of ozone can be even shorter.
2. The rate of ozone decomposition in water increases as pH increases. At low pH (< 7) and in the absence of UV radiation or hydrogen peroxide, the predominant species is molecular ozone, O_3. However, as pH increases, the rate of decomposition of ozone to produce the very short-lived (micro seconds) hydroxyl free radical (HO) increases. Thus above pH 9, and particularly as the pH approaches 10, very little molecular ozone can be detected in solution compared to the amount added.
3. Bicarbonate and carbonate anions are excellent scavengers (destroyers) of hydroxyl free radicals. In fact, carbonate ion is approximately 27 times more effective in scavenging hydroxyl free radicals than is bicarbonate ion. Consequently, when the alkalinity of recycling cooling water is high (> Ca 200 mg/ℓ) and the pH also is high (> Ca 9), the addition of ozone results in very little benefit. Ozone decomposes rapidly to hydroxyl free radicals (at elevated pH) which are lost even faster from the system by reaction with bicarbonate and carbonate anions.
4. When bromide ion is present in water being treated with ozone, it is quickly oxidised to hypobromite ion/hypobromous acid. This

"free bromine" can (and probably does) provide some of the biofouling control usually attributed to ozone alone. (This aspect will be discussed in greater detail later).

5. Some inorganic materials react very rapidly with ozone, particularly sulphide, nitrite, bromide, iodine and cyanide ions However, most inorganic materials cannot react with ozone (calcium, magnesium, silica, carbonate, bicarbonate, sulphate). Therefore, since some amounts of these materials are removed from solution when cooling waters are treated with ozone, their removal must be a result of solubility limitations or secondary reactions.
6. Even though some organic materials react rapidly with ozone (non-chlorinated alkenes and phenols), organic materials are rarely converted completely to carbon dioxide and water. Normal cooling tower ozonation conditions are not sufficient to convert most organic compounds present completely to CO_2 and water. Most organic materials react only slowly with ozone (many aromatics, chlorinated organics, low molecular weight acids, aldehydes, mixed aldehyde-acids, etc.). Conditions which favour the production of hydroxyl free radicals (pH 8 - 9; low alkalinity) also favour the more complete oxidation of most organic materials.
7. Since organic compounds form carbon dioxide only rarely, other (organic) "end products of oxidation" must be produced, particularly under cooling water ozonation conditions. These ozone end products generally are low molecular weight aldehydes, carboxylic acid, ketones and mixed aldehyde-acids. The most stable organic end product of ozone oxidation of organic materials is oxalic acid. This compound is unaffected by molecular ozone, but can be converted to CO_2 and water by hydroxyl free radicals.
8. Hypochlorite ion is oxidised by ozone to produce chloride ion (77%) and chlorate ion (23%). On the contrary, hypochlorous acid (HOCl) is unaffected by ozone. Therefore, "free chlorine" at pH well above 7.0 (at which it is primarily in the hypochlorite ion form) will be destroyed during ozonation.
9. Monochloramine is also destroyed by ozone, but more slowly, forming chloride ion and nitrate ion.
10. Primary oxidation products formed by ozonation may result in the formation of secondary oxidation (eg. the formation of (OBr)/HOBr (free bromine) when ozone oxidises bromide ions; the precipitation of calcium ion as $CaCO_3$ or as an insoluble organics).

Any or all of these reactions may occur simultaneously. In practice, however, one or the other reaction may predominate, depending on the reaction conditions and the chemical make up of the water being treated with ozone.

The two salient points that came out of a consideration of ozone chemistry are first of all the quality of the water particularly in respect of alkalinity, pH and the concentrations and compositions of the organic and inorganic matter contained in the water. Secondly the technique of ozone application is important. As with chlorine a choice has to be made between continuous dosing or intermittent dosing. A compromise between costs and biocidal activity will be necessary; much will depend on the water quality.

4.3 AMINES

A wide range of different compounds may be included under this general heading covering straight chain amines to quarternary compounds. These nitrogen containing compounds are surface active. The general structure of these cationic biocides is shown below:

$$\left[\begin{array}{c} R_4 \\ R_3 - N - R_1 \\ R_2 \end{array} \right] - \text{halide}$$

"R" represents alkyl, aryl or heterocyclic radical substitute containing 8 to 25 carbon atoms bonded to a nitrogen atom.

Quarternary compounds are generally most effective against algae and bacteria in alkaline pH ranges. Their biostat action is attributed to their cationic charge, which forms an electrostatic bond with the negatively charged sites on the microbial cell wall. The electrostatic bonds create stresses in the cell wall, causing cell lysis and death. The chemicals also cause cell death through protein denaturation, by distorting the permeability of the cell wall, which reduces the normal flow of life-sustaining nutrients into the cell.

The cationic character imparts a dispersant quality to the additive, but at the same time, could lead to foaming in cooling towers and open channels. A further complication in their use may be as a result of reaction with other additives such as scale preventatives and corrosion inhibitors. The activity of these chemical additives falls off rapidly in systems heavily fouled with dirt, oil and debris. The surface activity also promotes emulsification of any oil contained in the system which can give rise to additional operating problems, and loss of biocidal activity. In general amines do not present effluent discharge difficulties.

4.4 ORGANO SULPHUR COMPOUNDS

A variety of organo-sulphur microbiocide formulations are available for use in cooling water systems, and although their mechanisms of microbiocidal activity are similar, the pH ranges affecting their application are quite different.

In general, the organo-sulphur compounds function as microbiocides by either competitively or non-competitively inhibiting cell growth. The competitive inhibition action of organo-sulphur compounds closely resembles that of chelating agents. Normally, in microbial respiration, a low energy ferric (Fe^{3+}) cytochrome, accepts an electron and is transformed to a high energy, ferrous (Fe^{2+}) cytochrome state. These reactions result in the generation of energy required for life.

The competitive-inhibiting type of organo-sulphur compounds remove the ferric (Fe^{3+}) ion from the reaction by complexing it as an iron salt. Removal of the iron ion from the cytochrome stops the transfer of energy and causes immediate cell death.

Non-competitive inhibition caused by certain organo-sulphur compounds

consists in its elementary form, of nothing more than inducing the micro-organism to accept a chemical substance that will eventually lead to its destruction. Microbial death is brought about by acceptance of an organo-sulphur compound, sufficiently similar in structure to the essential metabolite that it will combine with the appropriate enzyme protein, but sufficiently different to ensure it will not produce the required life-sustaining reaction.

Organo-sulphur compounds are generally less sensitive to pH than some other biocides, but their cost may limit their general application.

Methylene bisthiocyanate is an organo-sulphur compound effective in inhibiting algae, fungi and bacteria, most notably the sulphate reducing species. Since it is a competitive inhibiting microbiocide, it inactivates the electron transfer cytochromes of the micro-organism. The thiocyanate fragment, $NCS-CH_2-SCN$, of the methylene ester of thiocyanic acid reacts to block the transfer of electrons in the micro-organism, resulting in cell death.

Although methylene bisthiocyanate is a very effective microbiocide in cooling water systems, it is not very soluble in water and therefore is usually formulated with dispersants. The dispersants enable it to function as an effective microbiocide throughout the water system and enhance its ability to penetrate algal and bacterial slime layers.

Methylene bisthiocyanate is pH sensitive and rapidly hydrolyses in pH ranges above 8.0. For this reason it is not recommended for use in systems where the recirculating water pH generally exceeds 8.0.

Sulphones and thiones are non-competitive organo-sulphur microbiocides. In water, they decompose to form solutions similar to microbial metabolites, but which are actually metabolite analogues and consequently cause cell death. As with all organo-sulphurs, they are pH sensitive, sulphones are most reactive in cooling water in the range of 6.5 - 7.5, and thiones in the pH range of 7 - 8.

Sodium dimethyl dithiocarbamate and disodium ethylene bisdithiocarbamate are organo-sulphur compounds which possess excellent microbiocidal capabilities. They are readily soluble in water and function best at pH 7 and above.

5. Other techniques for dealing with biofilms

Although the use of biocides is common industrial practice other techniques may be employed (see Table 4).

TABLE 4. Non-biocidal treatment of cooling water

Method	Problem associated with the technique
High temperature sterilisation. Heat shock treatment.	May give rise to large lumps of biomass in subsequent cooling water flow.
Radiation sterilisation, eg. ultra violet light and gamma rays.	Optical access is needed requiring transparent sections. For large systems the capital cost may be prohibitive. The radiation, in general, only affects the organisms it "sees" in the water, ie. generally not on surfaces. Gamma rays also require elaborate safety precautions.
Osmotic shock, ie. passing salt water through a normally fresh water system	Long time scale required. Corrosion may be encouraged.

In general these methods are not always effective compared with biocides and may be difficult to apply.

6. Concluding Remarks

A brief review of the application of biocides to combat the effects of biofilms in industrial equipment cannot discuss all the relevant points. The review however, does demonstrate the complexity of the technology and highlights the principal aspects of treatment.

In order that a proper choice of biocide may be made a complete analysis of the water will be necessary. Even constituents in small concentration may greatly affect the efficacy of a particular biocide due to chemical reactions. The level of pH in the system is another important factor that can affect the stability of a biocide, and pH is likely to be different in different parts of the system. The pH may also be different in the vicinity of the biofilm compared to that measured in the bulk. A proper understanding of the chemistry of the biocide and the likely pH changes in the system will be necessary before an informed choice may be made.

In order to obtain good disinfection and biofilm removal attention to flow velocities across a biofilm will be important. High velocities favour removal but the additional cost of pumping involved will need to be taken into account in the assessment of costs.

It is essential that the choice of biocide will also take into account problems of pollution and the protection of the environment. It is certain that these considerations will ultimately increase the cost of

treatment so that it is imperative that the technology adopted will be reasonably effective at minimum cost.

7. References

APV (1982). *Cooling water fouling in plate heat exchangers.* Spearhead APV Co. Crawley, Sussex.

Betz Laboratories Inc. (1976). Betz Handbook of Industrial Water Treatment. 7th Edn. page 204, Betz Laboratories, Trevoise, USA.

Bott, T.R. (1988). *Fouling in cooling water systems.* Data Item 88024, ESDU Intl.

EPRI (1980). *Biofouling control with ozone at the Bergen Generating Station,* EPRI Research Report CS 1629.

EPRI (1985). *Condenser-targeted chlorination design.* EPRI Research Report CS 5279.

Flemming, H.C. (1991). *Biofouling in water treatment* in Flemming, H.C. and Geesey, G.G. Eds. Biofouling and biocorrosion in industrial water systems. Springer-Verlag, Berlin.

Garey, J.F. (1979). *Biofouling control investigations 18 month summary report.* EPRI Project Interim Report EA 1082.

Kaur, K., Leadbeater, B.S.C. and Bott, T.R. (1988). *Ozone as a biocide in industrial cooling water treatment.* Proc. Int. Conf. Fouling in Process Plant. Oxford.

Marshall, A. and Bott, T.R. (1988). *Effectiveness of biocides* in Melo, L.F. et al editors. Fouling Science and Technology, page 591. Kluwer Academic Publishers.

Nesaratnam, R.N. and Bott, T.R. (1984). *Effects of velocity and sodium by hypochlorite derived chlorine concentration on biofilm removed from aluminium tubes.* Heating and Ventilating Engineer, 5-8 No. 664, 5.

Rice, R.G. et al (1981). *Uses of ozone in drinking water treatment.* Journal AWNA 73, 44.

Rice, R.G. and Wilkes, J.F. (1991a). *Fundamental aspects of ozone chemistry in recirculating cooling water systems - data evaluation needs.* International Ozone Conference. Pan American Committee, Toronto.

Rice, R.G. and Wilkes, J.F. (1991b). *Fundamental aspects of ozone chemistry in recirculating cooling water systems.* Paper No. 205. Conf. Corrosion 91. Nat. Assn. Corrosion Engineers, Houston.

BIOCIDE EFFICACY ON BIOFILM GROWTH

M.D. PUJO AND T.R. BOTT
School of Chemical Engineering
University of Birmingham
Edgbaston
Birmingham B15 2TT. UK

1. Introduction

The importance of controlling microbial growth in recirculating water cooling systems as well as in other processes such as paper mills, is vital in order to increase productivity and maintain operational efficiency of the process.

Cooling towers and paper mill systems are ideal environments for the growth of slime forming bacteria and fungi, as in such systems many nutrients are available and also the conditions of temperature and pH are often optimum for micro-organism growth.

The most common way to prevent slime formation in aqueous systems is the application of biocides, that may be combined with dispersants or surfactants which aid in breaking up accumulations of slimes and therefore help to improve performance.

Chlorine is commonly used for controlling biological fouling but large quantities will be required if there is a large chlorine demand in the system, ie. reaction with organic compounds in the water. Also, chlorine does not easily disperse slimes formed on a surface, or to react with bacterial growth underneath the slimy deposits.

Therefore, other biocides must be considered and are of two types: (1) oxidising biocides which comprise most of the halogenated compounds and halogens and (2) non-oxidising biocides which are either enzyme poisons that can block the transfer of electrons in the respiration enzymes or cell and cytoplasm damaging biocides that are toxic to micro-organisms by virtue of their surface activity.

This work consists of the study of the specificity of the effectiveness of a proprietary biocide in laboratory cooling water models, simulating industrial conditions.

2. Experimental Apparatus and Procedure

The two laboratory models used in the experimental work were for the simulation of an industrial water cooling system. The first model was composed of two loops, a "control loop" and a "biocide loop", this latter being fed from the "control loop", the two recirculating water

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 583–588.

systems remaining independent. The biocide was inserted only in the "biocide loop". Seven test sections were incorporated in the apparatus, five in the "control loop" and two in the "biocide loop".

The general arrangement of the equipment and flow circuits are shown in Figure 1.

The second laboratory model consisted of a single loop composed of four test sections, similar to that of the first rig.

In order to maintain the respective flowrates to the test sections in both sets of apparatus, each of the different streams passed through a control valve and rotameter. After passing through the test sections, the solution flowed back to the mixing vessel, to be recirculated. An overflow ensured a constant volume in each mixing vessel.

The water used for the biofouling system was distilled water to avoid problems with the variability of tap water quality and to ensure initial water sterility.

A pure culture of *Pseudomonas fluorescens* was used as the contaminant. The *Pseudomonas fluorescens* was grown by continuous culture in a 5 litre fermenter and at a dilution rate of 0.02 h^{-1}.

3. Analytical Methods

Biocide treatment programmes were evaluated by infra-red devices that measured the light absorption of the biofilm. The degree of absorption represents a regime of biofilm "density", ie. a combination of thickness and compactness.

4. Results and Discussion

Three experiments were carried out in order to study the effect of the biocide on biofilms, grown under specific conditions summarised in Table 1.

TABLE 1. Conditions of the different experiments

Experiment	Fluid velocity (ms^{-1})	Reynolds number (RE)
A	0.8	16.500
	1.5	20,625
B	0.8	16,500
	1.5	20,625
C	0.7	7,300
	1	10,435
	1.5	15,655

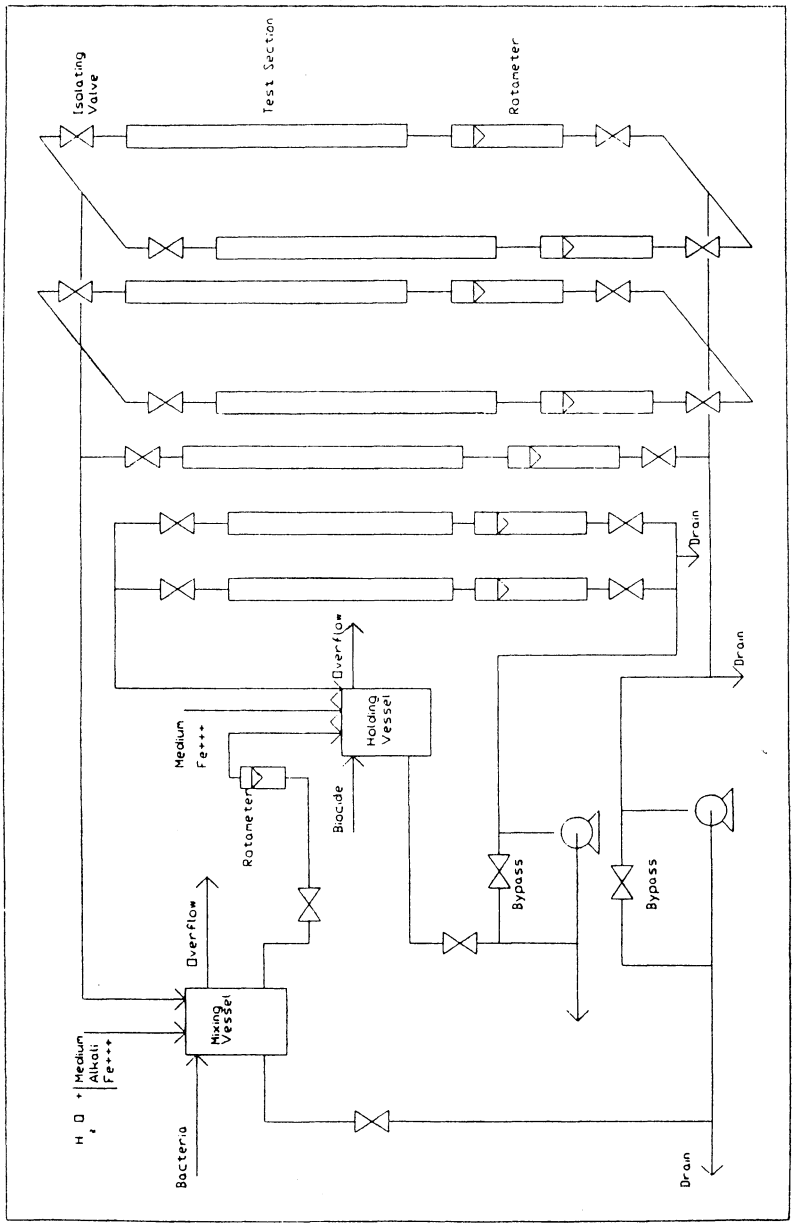


Figure 1. Flowsheet of overall biofouling rig with additional biocide loop

In the calculation of the Reynolds numbers, the physical properties were based on those for water at the circulating temperature.

Figures 2, 3 and 4 illustrate the effect of the biocide on established biofilms, respectively grown under the conditions of Experiments A, B and C.

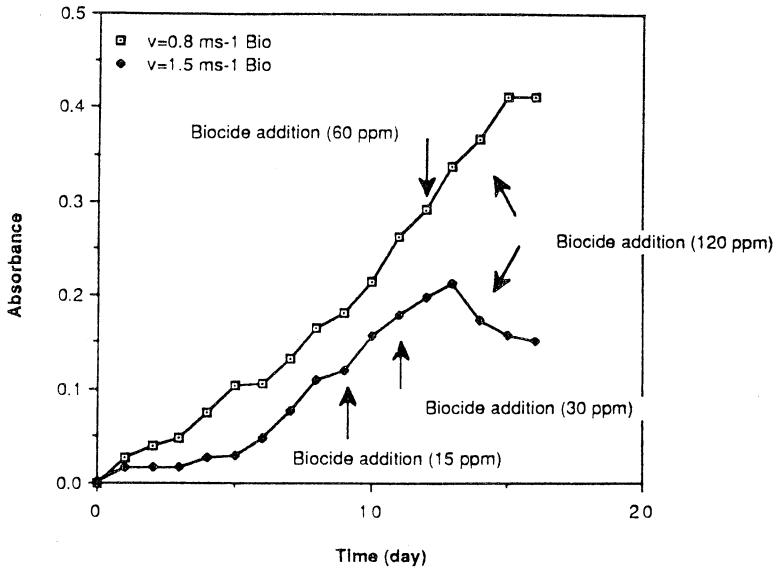


Figure 2. Biofilm growth in the biocide loop at Re = 16,500 (v = 0.8ms^{-1}) and Re = 20,625 (v = 1.5 ms^{-1})

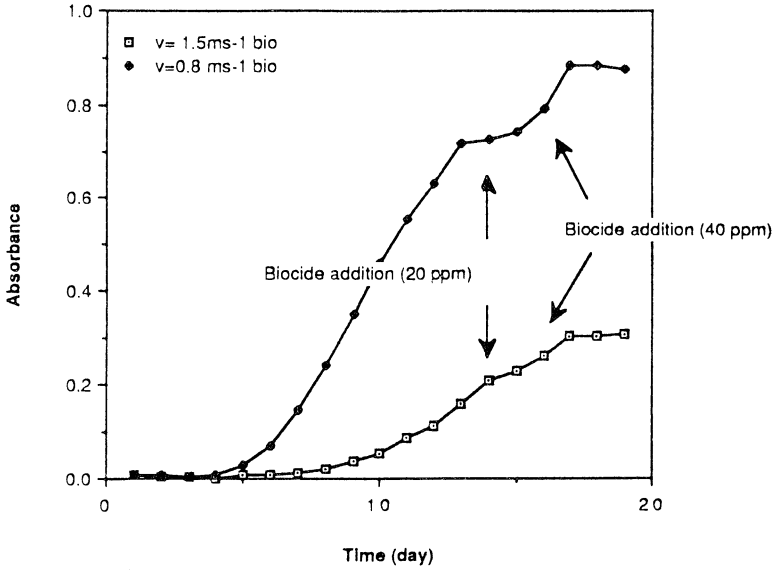


Figure 3. Biofilm growth in the biocide loop at Re = 16,500 (v = 0.8ms^{-1}) and Re = 11,000 (v = 1.5 ms^{-1})

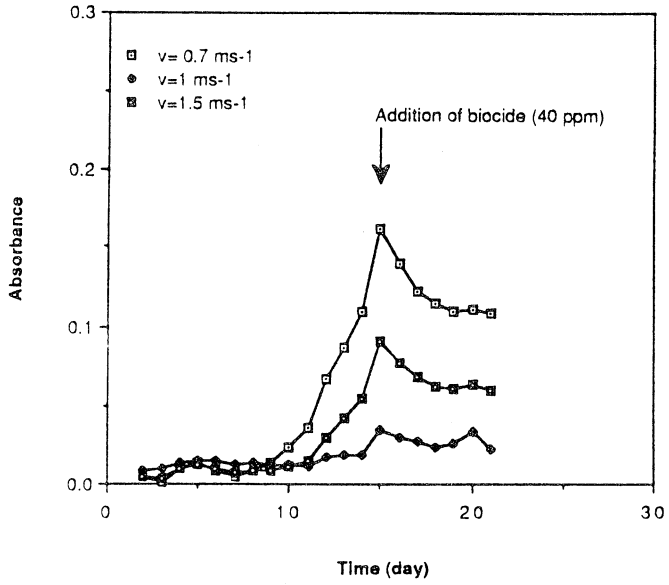


Figure 4. Biofilm growth at different fluid velocities

The biocide was added continuously, and the concentrations of biocide (as ppm of the circulating water of prepared biocide) were varied to study the effect on established biofilms.

As shown in Figures 2 and 3, continuous additions of biocide at low concentrations (15, 20 and 30 ppm) does not seem to be very effective, on biofilms already well established. Such concentrations appear to be inadequate to impede further biofilm growth.

Although a concentration of 40 ppm is not very satisfactory when applied on well developed biofilms (Figure 3) such concentration allows a reduction in absorbances, by about a quarter, four days after the addition of the biocide, on biofilms that had just started to form.

A higher concentration of 60 ppm seems to be more successful at higher velocity (Figure 2), probably due to a greater rate of diffusion of biocide (as well as nutrients) to the deposit resulting from the higher turbulence levels.

However, a concentration of 120 ppm stops the developed biofilm from increasing further at low velocity ($v = 0.8\ ms^{-1}$) (Figure 2); the mass transfer of biocide to the biological deposit is high enough to stabilise microbial growth but not to lower it, whereas at high fluid velocity ($v = 1.5\ ms^{-1}$), the biofilm formation decreases.

5. Conclusion

The results suggest that a continuous addition of biocide at low concentrations (20-40 ppm) on well established biofilms, does not affect those biofilms. However, such concentrations seem to be sufficient to

prevent any further biological growth, when used at the early stage of biofilm development. Higher concentrations (60 - 120 ppm) are more effective as expected, particularly at high fluid velocities at which the mass transfer of biocide is greater.

6. Acknowledgements

The authors would like to record their thanks to Grace Dearborn Ltd. for the provision of funds to enable this work to be carried out. The technical help provided by Michelle Cowbourne is also gratefully acknowledged.

EFFECT OF OZONE ON *PSEUDOMONAS FLUORESCENS*

K. KAUR and T.R. BOTT
School of Chemical Engineering
University of Birmingham
Edgbaston
Birmingham B15 2TT. UK

B.S.C. LEADBEATER
School of Biological Sciences
University of Birmingham
Edgbaston
Birmingham B15 2TT. UK

1. Introduction

To combat the problem of biofilm formation in cooling water systems biocides are used and chlorine has been most commonly employed. Legislative restrictions on effluent chlorine residuals and concern over toxicity from its reaction products requires that chlorine be added judiciously for biofouling control. Experimental data on the use of chlorine as a biocide has been presented by Miller (1982) and Nesaratnam (1984).

Ozone has been used for disinfection of municipal water supplies since the beginning of the century in Europe and is known to be an even more powerful disinfectant than chlorine. Ozone has been tested as an alternative method for treating a cooling water system, with success by H. Banks Edwards (1983).

The primary objective of this paper is to summarise and highlight the results from an experimental system (Kaur *et al*, 1989; Kaur *et al*, 1991), which indicate the areas of concern relating to the possible full-scale application of ozone for commercial cooling water treatment.

2. Materials and Methods

The laboratory system used for biofilm development consisted of glass test equipment generally as described by Pujo and Bott (1991). The basic principle of the biofouling system is that water composed of nutrients, bacteria (*Pseudomonas fluorescens*) and filtered mainswater (using carbon and 1 μm pore size filters) is recirculated through glass test-sections; representing tubes of a heat exchanger.

As a biofilm developed in the recirculation mode, dissolved oxygen, pH, temperature, glucose and bacterial concentrations in the fouling fluid were measured. Biofilm growth was monitored by wet weight measurements, by visual and microscopy assessments. Wet weight values were converted to mean mass per surface area of the test-section, to give biomass as mg/cm^2, since the test-section weight and surface area

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 589–594.

were known before the start of the experiment.

Table 1 gives the conditions under which biofilms were produced. The average biomass at the "plateau" stage of biofilm formation was around 9.7 mg/cm^2.

Ozone was generated from dry air in a "Labo" ozoniser supplied by Ozotech Ltd. and absorbed in a specially designed contact vessel to produce residual ozone concentrations in water of 0.1-0.5 mg/ℓ.

The ozonated water at a controlled ozone residual was applied to the biofilms in the test-sections using the once through mode of the apparatus, to assess the effectiveness of this system for biofilm removal. Some test-sections were operated simultaneously in the recirculation mode with no ozone applied; these were the controls for the experiment. Following completion of these tests, biofilm regrowth was monitored by allowing the ozone treated test-sections to be operated once again in the recirculation mode of the apparatus.

Ozone gas was absorbed in distilled water to obtain an ozone-rich solution. A suspension of *P. fluorescens* cells were taken from the continuous culture and diluted with the ozonated solution. This was incubated for approximately 10 minutes and ozone residual was measured at regular intervals, at the end of this period samples were taken for viewing using electron microscopy and for viable counts.

TABLE 1. Standard biofilm growth conditions

Residence time of fluid in apparatus in recirculation mode	30 mins
Velocity of fluid through test-sections	1 m/s
Reynolds number	15000
Temperature	25-30°C
Glucose concentration at start of test	5 mg/ℓ
Bacterial concentration in fluid (cells/mℓ)	1 x 10^7
Residual chlorine	<0.1 mg/ℓ
pH	7

3. SINGLE APPLICATIONS

From the results of the single applications of ozone to biofilms, the importance of biofilm quality or morphology was revealed for the flow velocity studied.

The results have indicated, firstly that as the age of the biofilm increases the amount of biofilm removed by a similar ozone residual, decreases. From this it appears that the older biofilm is more resistant to the effects of ozone, possibly because it has had a longer period of adjustment to the conditions used for development. Therefore, a long period of adjustment is likely to produce a more resistant biofilm.

Secondly, the results reveal that the greater the initial biomass, the lower the rate of removal using similar ozone residuals. This suggests that rate of diffusion of ozone through the biofilm is an important factor in controlling rate of removal using ozone.

Finally, biofilms that are adapted to conditions of high (>0.1 mg/ℓ) total chlorine in the mainswater entering the cooling water apparatus, require a higher ozone residual for biofilm removal as compared to biofilms grown in conditions of low chlorine levels. This may be explained by a more resistant nature of the biofilm produced under higher chlorine levels. Under these circumstances, the biofilm is likely to be less prone to removal by other oxidising biocides, such as ozone.

3.1 EFFECT OF FLUID VELOCITY

As the fluid velocity is increased the mass transfer of ozone to the biofilm will be increased due to the higher turbulence levels and a reduction in the mass transfer resistance a the interface. In addition as velocity increases, the shear at the biofilm/fluid interface also increases. Tests were carried out to investigate the effect of velocity on biofilm removal using a fixed ozone residual over a range of fluid velocities. In order to avoid problems of interpretation, biofilms were grown under the velocity conditions at which the ozone was subsequently to be applied. Results of one such test are shown in Figure 1.

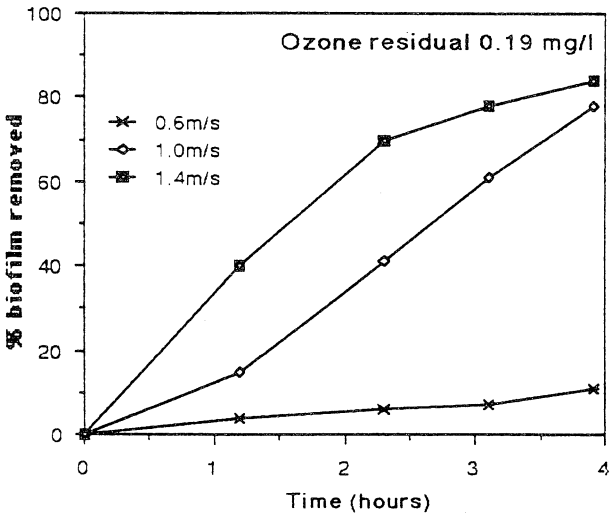


Figure 1. Effect of fluid velocity on biofilm removal

The general pattern observed is that the initial rate of biofilm removal is greater at the higher velocities and this may be attributed

to a combination of relatively high mass transfer and relatively high shear at the biofilm surface.

3.2 INTERMITTENT DOSING

In full-scale operation, for economic reasons, it is likely to be advantageous to use intermittent dosing of cooling water circuits with ozone. Some preliminary tests to determine the effects of the intermittent regime for ozone application have been performed. These tests involved developing biofilms under standard conditions of growth and then applying ozonated water at set residuals for a certain duration (the ozone dose). Following ozone treatment, the biofilms were returned to the recirculation mode of the apparatus and no bacteria were added from the continuous culture, after regrowth for 40-65 h a further ozone dose was applied, this intermittent treatment procedure was used to evaluate the effectiveness of various ozone doses.

The results of one such experiment are shown in Table 2, where the fluid velocity was 1 m/s (ie. a suitable velocity for cooling water in heat exchangers).

TABLE 2. Intermittent dosing results

Dose no.	Ozone residual (mg/ℓ)	Duration of ozonation (hours)	Initial removal rate (%/min)	Regrowth biomass after 40 h (mg/cm^2)
1	0.06	3	1.56	2.24
2	0.04	2	1.35	13.98
3	0.03	3	0.93	4.68
4	0.02	3	1.30	5.09

It is clear that the low ozone residuals have been effective in virtually complete removal of these biofilms in periods of up to 3 h. Following dose 1 and 2, 93% of the biofilm had been removed, after dose 3 only 73% and following dose 4 approximately 88%. It also has to be recognised that rapid regrowth can take place following the dosing period. However, the regrowth appears to be dependent upon the ozone residual and the duration of the dose, ie. the higher the ozone residual concentration applied and the greater the duration of the application the lower the rate of regrowth. Therefore, viability of bacteria remaining attached to the surface following an ozone dose can be affected and regrowth can be limited by adopting an effective combination of residual concentration and duration of the dose.

3.3 EFFECT OF OZONE ON *PSEUDOMONAS FLUORESCENS* IN SUSPENSION

The information obtained on the effect of ozone of bacterial cells indicated the presence of many membrane bounded vesicles, which could be

seen budding from the membrane of the treated bacterial cell wall. The resulting lysis of cells with associated 'cytoplasmic degradation' suggest that ozone acts first on the structure of the cell wall. Therefore, this process may be responsible for affecting viability of bacteria within the biofilm and it may also be responsible for biofilm removal. Ozone residual concentrations maintained at or above 0.4 mg/ℓ for 2-3 minutes were effective for a 100% kill, however concentrations of 0.1 mg/ℓ can also affect viability.

4. Conclusions

The use of ozone for *P. fluorescens* biofouling control, in the experimental cooling water system has been shown to be successful at low ozone residuals and for short dosing periods. However, the results have revealed that the quality or morphology of the biofilm can determine the effectiveness of the ozone dose.

The morphology of the biofilm is related to the growth conditions adopted during biofilm development, such as available nutrients, pH, residual chlorine and temperature. Variation in conditions such as chlorine residuals resulted in biofilms adapted to oxidising biocides, therefore requiring a higher ozone dose than biofilms developed under conditions of <0.1 mg/ℓ chlorine. Miller (1982) has indicated that the level of the available nutrients (carbon source) affects the type of biofilm developed; under conditions of high glucose concentrations a thicker biofilm is produced which is prone to sloughing. Therefore, a lower ozone dose would be required for removal of a biofilm developed under these conditions.

In general, biofilms in full-scale cooling water systems are variable in morphology. They are likely to be composed of a variety of microbial species and developed under variable growth conditions, such as low levels of carbon source (possibly in the region 1-7 mg C/ℓ), not all of which is biodegradable. The structure of these biofilms will also vary according to seasonal changes in the predominant microbial species the level of carbon and temperature.

In the experimental system as biofilm development progressed, the structure of the biofilm changed and an increase in biofilm thickness or mass was noted for the older biofilm. The age of the biofilm has been shown to determine the effectiveness of the ozone dose. The older biofilm has been shown to be less prone to removal by ozone. These results suggest that a commercial ozone dosing regime that could be adopted, whereby biofilms would be allowed to develop for a certain duration which then could be easily removed using low ozone doses. The success of this control method would require a full understanding of biofilm development and the duration of the growth period that could be endured between ozone doses.

The investigations of the influence of fluid velocity during ozone application has indicated that, an increase in fluid velocity results in increased biofilm removal rates. However, the fluid velocity in the full-scale situation will be limited and could not be easily varied, but the ozone application system could be installed with the capacity to take advantage of using higher fluid velocities, that are required to

improve ozone transfer and biofilm removal. The capital and running costs of such a system would have to be compared to the possibility of using higher ozone doses to achieve similar results.

The experimental results produced for intermittent application of ozone underline the importance of adopting an effective ozone dose that is capable not only of biofilm removal but affects the viability of attached bacteria. Adopting an effective intermittent regime can limit biofilm regrowth potential. The potent biocide action observed on bacterial cells in suspension has indicated that ozone can be used for affecting viability of cells, even at ozone concentrations of 0.1 mg/ℓ. Intermittent ozone dosing appears to be the most economical system that could be installed in a full-scale application for biofouling control. However, the ozone dose has to be tested in relation to a particular system and controlled to prevent inducing increased biofilm regrowth following treatment.

In conclusion, the results from this experimental system have shown the complexity of the relationship between the morphology of the biofilm and the biocide action of ozone. It is likely that the success of ozone application to commercial cooling water systems will be site specific and pilot investigations of the morphology of the biofilms would provide the basis for the installation of full-scale ozone treatment. The preliminary results from the use of an intermittent ozone dosing regime indicates, that this method has the potential for the most economical and effective treatment for controlling biofouling in commercial cooling water systems.

5. Acknowledgments

The authors would like to express their gratitude to SERC for the provision of an award and Ozotech Ltd. for financial and technical assistance. The contribution of A. Curran is also recorded.

6. References

Edwards, H.B. (1983). *The economical advantage of ozonating the cooling tower water system.* International Ozone Association 6th World Congress, Washington DC, USA. May.

Kaur, K., Leadbeater, B.S.C. and Bott, T.R. (1989). *Ozone as a biocide in industrial cooling water treatment.* International Ozone Association Conference, Wasser Berlin, W. Germany, Berlin. 10-16th May.

Kaur, K., Curran, A. and Bott, T.R. (1991). *The effect of ozone on Pseudomonas fluorescens.* International Ozone Association 10th World Congress, MOnaco, 19-21st March.

Miller, P.C. (1982). *Biological fouling film formation and destruction.* In PhD thesis, University of Birmingham, Chemical Engineering Department, UK.

Nesaratnam, R.N. (1984). *Biofilm formation and destruction on simulated heat transfer surfaces.* In PhD thesis, University of Birmingham, Chemical Engineering Department, UK.

DISINFECTANT TESTING USING FOODBORNE BACTERIA IN BIOFILMS

G. WIRTANEN and T. MATTILA-SANDHOLM
VTT Food Research Laboratory
P.O.Box 203, SF-02151 Espoo, Finland

1. Introduction

Biofilm is a phenomenon which develops in several branches of industry, leading however to similar consequences such as increased resistance against antibiotics and disinfectants. In medicine biofilm is called glycocalyx (Costerton *et al.*, 1981a, 1981b). In processing equipment and circulation systems biofilm protects the microbes against cleaning and sanitation measures. The biofilm layers also trap nutrients, which is an important factor when bacteria grow on surfaces and the nutrient acquisition is limited (Bott *et al.*, 1983; Costerton *et al.*, 1987; Characklis and Marshall, 1990). These and other results indicate that sanitizer and process disinfectants intended for use in the process industry should be tested on surfaces with test strains important for the industry in question (LeChevallier *et al.*, 1988; Czechowski, 1990; Frank and Koffi, 1990). However, sensitivity tests of antibiotics and disinfectants against microbes are still generally performed using suspension cultures, which normally do not form biofilm (Anwar *et al.*, 1990). Results achieved in such trials do not correspond with practical situations and they have been called "interesting laboratory exercises" by Costerton *et al.* (1981b). Biofilm consists of both microbes and surrounding polysaccharides (Bott *et al.*, 1983). The formation of biofilm can be induced using low-nutritive substrates, using antibiotics, by stressing the microbes, and above all using surfaces instead of suspensions (Costerton *et al.*, 1981a, 1981b, 1987; Miller and Bott, 1982).

2. Materials and Methods

2.1. TEST ORGANISMS AND TEST MATRIX

Microbes used in these tests were *Listeria monocytogenes* ELI 4002, *Pseudomonas fragi* ATCC 4973 and *Pseudomonas fluorescens* ELI 96. The organisms were cultivated on trypticase soy agar (TSA, BBL, U.S.A.) and stored at 4 °C. Fresh suspensions of cultures containing vegetative cells were grown for 18 h (overnight) in Iso Sensitest broth (ISB, Oxoid, UK) or in tryptic soy broth (TSB, Difco, U.S.A.): *P. fragi* and *P. fluorescens* in ISB at 30 °C and *L. monocytogenes* in TSB at 37 °C. The inocula used contained about 10^7 - 10^8 cfu/ml broth. Autoclaved meat soup (Jalostaja, Finland) was used as a test matrix simulating the organic load in the test environment.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 595–600.

2.2. DISINFECTANTS

The disinfectants and sanitizers tested were: sodium hydroxide (NaOH) and hydrochloric acid (HCl, Merck) diluted in distilled water to 0.1 M and 0.05 M solutions; hypochlorite (Oy Ecolab Ab) consisting of 14 % free chlorine diluted in distilled water in concentrations of 1 % (pH 11.5) and 0.1 % (pH 10.5); iodophore (Oy Ecolab Ab) consisting of 6.3 % phosphoric acid diluted in distilled water to the ready-to-use concentrations 2 % and 0.2 %; a quaternary ammonium compound (Oy Ecolab Ab) consisting of 9 % alkyl dimethylbenzyl ammonium chloride diluted in distilled water to the ready-to-use concentrations 0.1 % and 0.01 %; and an anionic tenside (Finnewos Agri Oy) consisting of 60 % potassium peroxisulphate, 20 % anionic tenside and 5 % sulphamic acid diluted in distilled water to ready-to-use concentrations of 1 % and 0.1 %.

2.3. TESTING OF BIOFILM FORMATION ON SURFACES

Autoclaved meat soup was used as a test matrix to obtain a natural organic load in the test environment. *P. fragi* was allowed to grow initially for 24 h and *L. monocytogenes* for 48 h on steel surfaces (AISI 316). Thereafter the meat soup medium was changed in order to leave only the sessile organisms and the biofilm of each microbe was allowed to develop for 96 h at 25 °C. The incubated surfaces with biofilm developed were lifted off and rinsed in sterile saline. The alkaline-acid treatment was performed with NaOH for 5 min and with HCl for 10 min at 25 °C. The hypochlorite, iodophore and anionic tenside treatments were performed at 25 °C for 30 min and the quaternary ammonium compound treatment at 37 °C for 30 min (Wirtanen and Mattila-Sandholm, 1992a, 1992b).

2.4. SUSPENSION TEST

The different disinfection and sanitation treatments were also carried out in suspension tests. The suspension contained 2 ml bacterial suspension (approximately 10^4 cfu ml^{-1}), 2 ml meat soup and 2 ml disinfectant solution at the appropriate ready-to-use concentration. The meat soup was added as an organic load. The treatment for each sanitizer was identical to that used in the surface test (Wirtanen and Mattila-Sandholm, 1992a, 1992b).

2.5. MICROBIAL CULTIVATION

The biofilm bacteria were scraped from the test surfaces and then suspended in inactivation solution. The inactivation was allowed to act for 5 min at 45 °C. In the suspension test the liquid sample was mixed with inactivation solution and diluted as a logarithmic series in saline. The microbes were cultivated on TSA plates in duplicate: *L. monocytogenes* at 37 °C, *P. fragi* and *P. fluorescens* at 30 °C for 48 h (Wirtanen and Mattila-Sandholm, 1992a, 1992b).

2.6. QUANTITATIVE GLYCOCALYX DETERMINATION

For glycocalyx determination the samples were suspended in inactivation solution and centrifuged and resuspended in saline. The cells were then sonicated in an MSE sonicator (Scientific Instruments, UK). The polysaccharides in the supernatant were precipitated by adding supernatant in drops to absolute alcohol and the precipitate was collected by centrifugation. Thereafter the precipitate was dissolved in sulphuric acid, the reaction being cooled in an ice bath. Tryptophan (Sigma, US) was then added to each sample and the samples were boiled in a water bath and then

cooled on ice. The carbohydrate in the glycocalyx dehydrates in the presence of the concentrated acid to furfural. A brownish violet color developed as the condensation product of furfural with the aromatic amine tryptophan (Dall and Herndon, 1989). The absorbances were read in a 551S UV/VIS spectrophotometer (Perkin-Elmer, US) at 500 nm using a standard containing dextran (Sigma, US).

3. Results

The results of surface and suspension tests are shown in Figs 1-3. Overall, bacteria in the suspension tests showed greater sensitivity to the disinfectants and sanitizers than those tested in the surface tests. However, great variation existed between different agents and between the two strains used. The alkaline-acid treatment was evidently less effective on surfaces for both strains. The hypochlorite treatments, however, were equally effective both on surfaces and in suspensions. The results of the iodophore and quaternary treatments differed between the two strains. A clear difference was observed between the treatments in the case of *L. monocytogenes* but not with *P. fragi*. The anionic tenside was effective both on surfaces and in suspensions. The results presented

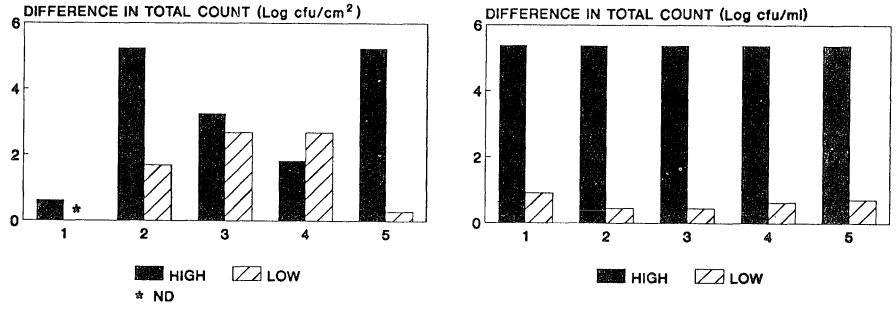


Figure 1. The effect of treatment with alkaline-acid (1), hypochlorite (2), iodophore (3), quaternary ammonium compounds (4) and anionic tenside (5) on *Listeria monocytogenes* in surface (left panel) and suspension tests (right panel). The results are expressed as logarithmic differences in bacterial counts compared with the untreated control. (ND = not detected)

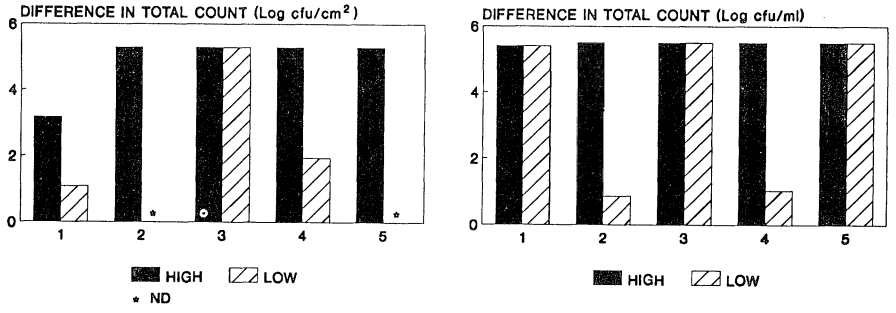


Figure 2. The effect of different sanitizer treatments on *Pseudomonas fragi* (see Fig. 1).

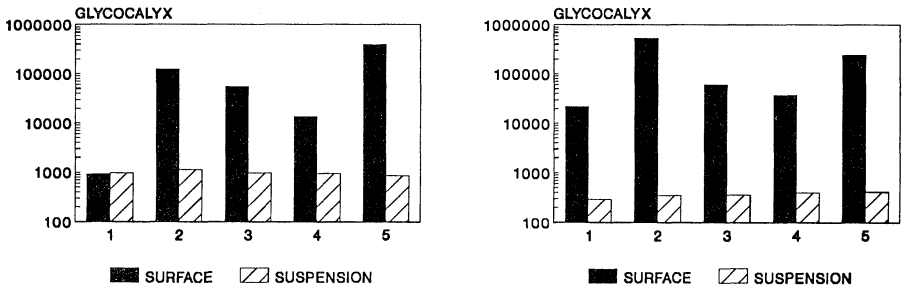


Figure 3. The amount of glycocalyx of *Listeria monocytogenes* (left panel) and *Pseudomonas fragi* (right panel) on the metal surface (AISI 316) and in the suspension culture after sanitizer treatment (see Fig. 1). The value is the ratio of glycocalyx per living cells between the treated and control samples.

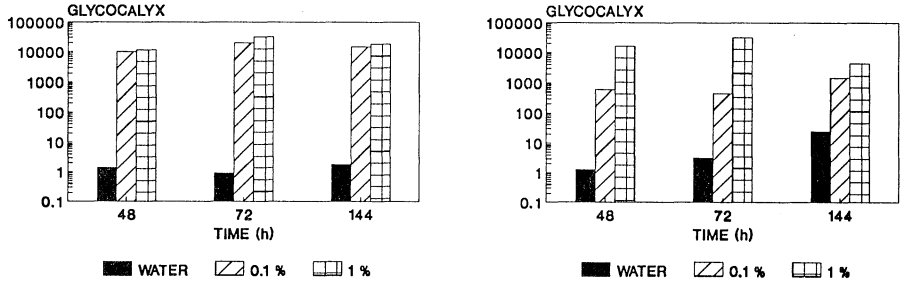


Figure 4. The amount of glycocalyx of *Listeria monocytogenes* (left panel) and *Pseudomonas fluorescens* (right panel) on the steel surface (AISI 316) after treatment with water and with hypochlorite in meat soup

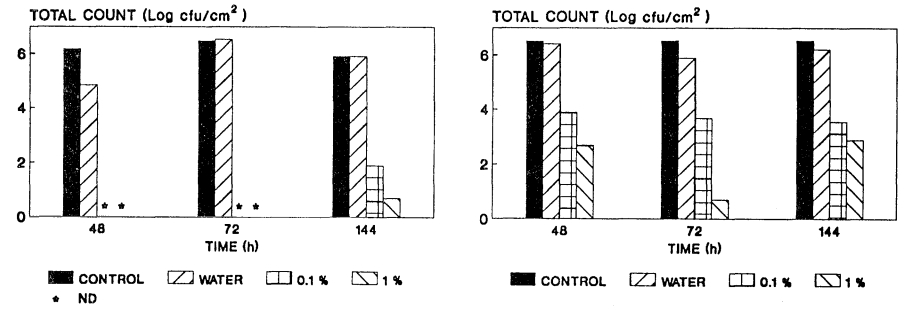


Figure 5. The effect of hypochlorite treatment on *Listeria monocytogenes* (left panel) and *Pseudomonas fluorescens* (right panel) biofilms at different growth phases in meat soup

in Figure 3 demonstrate that the amount of glycocalyx remaining on surfaces was much more evident than in suspension.

Figure 4 shows the biofilm build-up (glycocalyx) during a follow-up period of 144 h for *L. monocytogenes* and *P. fluorescens*. Preliminary trials showed that glycocalyx could not be measured after 24 h. The glycocalyx was demonstrated at 48 h and the figure shows that the glycocalyx residues per living cells were bigger on surfaces after hypochlorite treatment than on control surfaces with water treatment. It can be seen that *P. fluorescens* was more resistant than *L. monocytogenes*, although the amounts of glycocalyx formed by the two microbes did not differ significantly. Figure 5 shows the effect of hypochlorite treatment on growth on surfaces at different time intervals.

4. Discussion

Microbial resistance to antibiotics depends on the metabolic activity of the organisms. When microbes achieve the stationary growth phase they react totally differently than when they are growing actively (Brown *et al.*, 1988; Evans *et al.*, 1990). The effect of many antibiotics is based on inhibition of active growth. However, most of the bacteria in biofilms are no longer growing actively and their resistance is therefore higher. For this reason the resistances of different biofilm bacteria cannot be compared without controlling their growth rates (Gilbert *et al.*, 1990). Furthermore, in addition to the possible importance of bacterial growth rate other factors such as the importance of the permeability barrier may also be important from the point of view of resistance (Nichols, 1991).

In the present experiments the amount of glycocalyx produced by the strains tested remained very high after each disinfectant treatment (Wirtanen and Mattila-Sandholm, 1992a). Residual biofilm usually contains sufficient viable organisms for replication (Characklis and Marshall, 1990) and glycocalyx matrix of polyanionic nature probably contributes significantly to the biofilm resistance. Glycocalyx can also be rapidly created by surviving organisms as a protective response e.g. against chlorine (Characklis and Marshall, 1990). It seems likely that disinfectant treatments have been responsible for the high amounts of glycocalyx formed in some tests (Wirtanen and Mattila-Sandholm, 1992a, 1992b). However, contradictory results have also been obtained, indicating that chlorine may preferentially remove extracellular polysaccharides, thus leaving biofilm cells more exposed to nutrients when treatment ceases (Characklis and Marshall, 1990). The inactivation of *Listeria* and *Pseudomonas* strains both in suspensions and attached to surfaces has frequently been investigated (Herald and Zottola, 1989; Mustapha and Liewen, 1989; Best *et al.*, 1990; Shin-Ho-Lee and Frank, 1991). The results of these investigations lead to different conclusions partly because of variations in experimental procedures (Herald and Zottola, 1989; Mustapha and Liewen, 1989; Best *et al.*, 1990; Shin-Ho-Lee and Frank, 1991). The present results indicate that great variations exist between suspension and surface tests and that the amount of residual glycocalyx should be taken into account when developing sanitizers and disinfectants for use in the processing industry.

5. Acknowledgements

The authors thank BioOrbit (Finland) for providing the opportunity to use the ATP measurement equipment in this work. The technical assistance of Niina Kajander, Anne Karppinen and Raimo Matsson is gratefully acknowledged.

6. References

Anwar, H., Dasgupta, M. K. and Costerton, J. W. (1990) 'Testing the susceptibility of bacteria in biofilms to antibacterial agents', Antimicrob. Agents Chemother., 34, 2043-2046.

Best, M., Kennedy, M. E. and Coates, F. (1990) 'Efficacy of a variety of disinfectants against *Listeria* spp.', Appl. Environ. Microbiol., 56, 377-380.

Bott, T. R., Done, R. and Miller, P. G. (1983), 'Bacterial concentration and biofilm thickness' in Proc. Eng. Found. Conf. Fouling of Heat Exchanger Surfaces, United Engineering Trustees Inc., New York. pp. 735-740.

Brown, M. R. W., Allison, D. G. and Gilbert, P. (1988), 'Resistance of bacterial biofilms to antibiotics: a growth-rate related effect?', J. Antimicrob. Chemother., 22, 777-780.

Characklis, W. G. and Marshall, K. C. (1990) 'Biofilms', John Wiley & Sons, New York.

Costerton, J. W., Irvin, R. T. and Cheng, K.-J. (1981a) 'The bacterial glycocalyx in nature and disease', Ann. Rev. Microbiol., 35, 299-324.

Costerton, J. W., Irvin, R. T. and Cheng, K.-J. (1981b) 'The role of bacterial surface structures in pathogenesis', CRC Crit. Rev. Microbiol., 9, 303-338.

Costerton, J. W., Cheng, K.-J., Geesey, G. G., Ladd T. I., Nickel J. C., Dasgupta, M. and Marrie T. J. (1987) 'Bacterial biofilms in nature and disease', Annu. Rev. Microbiol., 41, 435-464.

Czechowski, M. H. (1990) 'Use of biocides to control biofilms', J. Food Prot., 53, 901-902.

Dall, L. and Herndon, B. (1989) 'Quantitative assay of glycocalyx produced by viridans group streptococci that cause endocarditis', J. Clin. Microbiol., 27, 2039-2041.

Evans, D. J., Brown, M. R. W., Allison, D. G. and Gilbert, P. (1990), 'Bacterial biofilms growth rate and susceptibility towards ciprofloxacin', in Proc. 90th Annu. Meet. Am. Soc. Microbiol. 1990, Am. Soc. Microbiol., Anaheim, Abstract A-151.

Frank, J. F. and Koffi, R. A. (1990) 'Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat', J. Food Prot., 53, 550-554.

Gilbert, P., Collier, P. J. and Brown, M. R. W. (1990), 'Influence of growth rate on susceptibility to antimicrobial agents: Biofilms, cell cycle, dormancy and stringent response', Antimicrob. Agents Chemother., 34, 1865-1868.

Herald, P. J. and Zottola, E. A. (1989) 'Effect of various agents upon the attachment of *Pseudomonas fragi* to stainless steel', J. Food Sci., 54, 461-464.

LeChevallier, M. W., Cawthon, C. D. and Lee, R. G. (1988) 'Inactivation of biofilm bacteria', Appl. Environ. Microbiol., 54, 2492-2499.

Miller, P. C. and Bott, T. R. (1982), 'Effects of biocide and nutrient availability on microbial contamination of surfaces in cooling-water systems', J. Chem. Technol. Biotechnol., 32, 538-546.

Mustapha, A. and Liewen, M. B. (1989) 'Destruction of *Listeria monocytogenes* by sodium hypochlorite and quaternary ammonium sanitizers', J. Food Prot., 52, 306-311.

Nichols, W. W. (1991), 'Biofilms, antibiotics and penetration', Rev. Med. Microbiol., 2, 177-181.

Shin-Ho-Lee and Frank, J. F. (1991) 'Inactivation of surface-adherent *Listeria monocytogenes.* Hypochlorite and heat', J. Food Prot., 54, 4-6.

Wirtanen, G. and Mattila-Sandholm, T. (1992a) 'Removal of foodborne biofilms - comparison of surface and suspension tests. Part I', Lebensm. Wiss. Technol., 25, 43-49.

Wirtanen, G. and Mattila-Sandholm, T. (1992b) 'Effect of the growth phase of foodborne biofilms on their resistance to a chlorine sanitizer. Part II', Lebensm. Wiss. Technol., 25, 50-54.

Chapter 10

LABORATORY METHODS AND INDUSTRIAL MONITORING

THE MEASUREMENT OF BACTERIAL ATTACHMENT TO SURFACES IN STATIC SYSTEMS

MADILYN FLETCHER
Center of Marine Biotechnology
Maryland Biotechnology Institute
University of Maryland System
600 E. Lombard St.
Baltimore, Maryland 21202
USA

1. Introduction

There have been many laboratory studies that have attempted to measure the adhesive properties of bacteria or their attachment to solid surfaces in laboratory systems. Many of these investigations have used "static" systems, which were still or gently agitated and where there was no directional flow. (Flow cell systems for evaluating adhesion are dealt with elsewhere in this volume.) These static system studies have employed a variety of designs, using numerous types of bacteria, substrata, and environmental conditions. They have usually been carried out in order to evaluate (a) the adhesiveness of specific bacteria, (b) the suitability of particular materials as attachment substrata, or (c) the influence of environmental factors on the attachment process. Some laboratory procedures can also be applied to studies in natural environments (e.g. water, soil, water delivery systems, cooling towers), but they must first be validated in the laboratory. Such methods include microcosopy, analysis of biochemical markers, and nucleic acid hybridization.

2. Experimental Design

2.1 INTRODUCTION

The numbers of bacteria that attach to surfaces and the rate of deposition depend on the species and strains of bacteria and on their nutritional status (e.g. growth phase, whether they are starved for specific nutrients). Attachment will also be influenced by environmental conditions, including nutrient sources and concentration and flux (Molin et al., 1982; Knox et al., 1985; McEldowney and Fletcher, 1986), electrolyte concentration (Marshall et al., 1971b; Ørstavik, 1977; Knox et al., 1985), pH (Gordon et al., 1981; Harber et al., 1983), and temperature (Fletcher, 1977; Harber et al., 1983).

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 603–614.

The characteristics of the solid surfaces are also extremely important, as can be the procedures used to rinse surfaces after attachment and before enumeration of attached cells. The results will depend heavily on the experimental design. Consequently, experimental variables must be evaluated and selected carefully, so that the design will address the specific question of interest. If the ultimate goal is to understand a natural process, it is extremely important that the experimental design is consistent with the natural system being modelled.

2.2 SELECTION OF ORGANISMS

The adhesive properties of bacteria depend upon their genetic capabilities and upon the modulation of those characteristics by their metabolic state. Also, laboratory culture can result in significant changes in adhesiveness of natural isolates with time, possibly because of selection of less adhesive strains by repeated transfer from liquid suspension. Recently, it has been clear that some bacteria are able to undergo genetic changes, termed phase variation, so that progeny express one of two, or more, adhesive phenotypes. For example, *Pseudomonas atlantica* (Bartlett et al., 1988) and *P. fluorescens* (Pringle et al., 1983) can both produce progeny of three separate colony types (with corresponding differences in adhesion ability), i.e., mucoid, smooth, and crenated colonies. With *P. fluorescens* H2, the crenated form is the most adhesive, whereas the smooth form is moderately adhesive, and the mucoid form, which produces an alginate exopolymer, is poorly adherent (Pringle et al., 1983; Pringle and Fletcher, 1983).

The polymers produced at the bacterial surface, not surprisingly, have a considerable influence on attachment. Bacterial species differ in their abilities to produce extracellular polymers, such as polysaccharide and protein fibrils (i.e. pili, fimbriae). There are also considerable differences in the compositions of extracellular polysaccharide, surface proteins, and lipopolysaccharide produced by different species and strains. The importance of the presence and compositions of these various surface polymers in the adhesion process can be demonstrated by experiments with mutants that are altered with respect to specific cell structures. Mutations in bacteria, such as *Escherichia coli* (cf. Isaacson, 1985) and *Streptococcus salivarius* (Weerkamp et al., 1986), that alter specific cell surface components can result in altered adhesiveness. For example, the attachment of *S. salivarius* to hydrophobic surfaces varied depending upon the density of a fibrillar layer on the cell surface and the properties and surface exposure of specific types of fibril (Weerkamp et al., 1987).

Growth conditions of bacteria can affect their adhesiveness, but there is no consistent pattern in such alterations (Marshall et al., 1971b; Molin et al., 1982; McEldowney and Fletcher, 1986). For example, in batch culture adhesiveness can vary with growth phase; however, there is no general rule as to whether cells are more adherent in exponential phase or in stationary phase (Harber et al., 1983; Rosenberg and Rosenberg, 1985).

Interactions among different species can also influence the outcome of attachment (McEldowney and Fletcher, 1987). This could be due to the production of metabolites that are released and subsequently influence the adhesion process through

physicochemical means (Fletcher, 1976; Gordon, 1987). Also, physiological interactions between organisms could result in changes in cell surface properties, in a manner analogous to alteration of surface characteristics by nutritional factors. There has been very little work investigating adhesion of mixed cultures and the possible effects of interactions on the attachment process. However, such studies will be necessary if we are to understand attachment phenomena in natural environments.

2.3. SUBSTRATUM COMPOSITION

Bacterial attachment can be markedly influenced by the physicochemical characteristics of the substratum. Surface properties that influence adhesion include electrostatic charge (Feldner et al., 1983), surface free energy (van Pelt et al., 1985; Busscher et al., 1986a,b) and a related parameter, hydrophobicity (Hogt et al., 1983; Ludwicka et al., 1984), and roughness (Baker, 1984).

For both theoretical and practical purposes, materials can be divided into two types: (1) high surface energy materials, which are hydrophilic, frequently negatively charged, and usually inorganic materials such as glass, metals, or minerals, and (2) low energy materials, which are relatively hydrophobic and low in surface charge and are generally organic polymers, such as plastics. High energy substrata readily adsorb dissolved solutes and atmospheric contaminants, and therefore are rarely clean. Thus, when using these materials as attachment substrata, it is particularly important that they are cleaned carefully and used immediately, before surface contamination can occur. Glass is commonly used as an attachment substratum, particularly because it is suited for subsequent microscopic examination. However, glass is rapidly contaminated by adsorbents in the atmosphere or solution, a factor that must be considered in experimental design and analysis of results.

Low energy materials are not contaminated so quickly as high energy surfaces. Frequently, these may be used directly from the supplier, such as with bacteriological or tissue culture plastic ware. A range of organic polymers (e.g., polystyrene, polycarbonate, polyamide) are available (Pringle and Fletcher, 1983), and a variety of studies have utilized such materials to evaluate the significance of particular functional groups and relative surface energies (Fletcher and Loeb, 1979; Pringle and Fletcher, 1983). Also, high energy materials such as glass can be derivitized with low energy compounds, such as by treatment with commercial siliconizing agents or by other chemically defined treatments (Satou et al., 1988) that confer, for example, amino, non-polar ethoxyl, hexyl, or carboxyl groups to glass surfaces.

3. Measurement of Bacterial Biomass or Numbers

3.1. MICROSCOPY

The first studies of bacterial attachment utilized light microscopy , and microscopy is still probably the single most valuable technique for evaluating bacterial attachment to surfaces. It not only allows determination of the numbers of attached cells, but also

provides information on their distribution. Moreover, in recent years, enormous advances have been made in the development and application of microscopic techniques to adhesion studies. These range from improvements in epifluorescent microscopy to image analysis and to confocal laser scanning microscopy.

Transmitted light microscopy (e.g., brightfield, phase) is commonly used to evaluate attachment to transparent surfaces. Frequently, the specimen is fixed and stained, e.g., with crystal violet, to better visualize the bacteria. The extracellular polymers associated with attached bacteria (which are probably also adhesives) can also be stained, e.g., with Congo red, so that polymer production can be evaluated (Allison and Sutherland, 1984).

The application of computer-enhanced microscopy and image analysis to studies of bacterial attachment has been extremely valuable. The tedious task of counting attached bacteria has been facilitated, and the area evaluated can be considerably increased within a given time. Image analysis systems have been used to determine numbers of cells attached, area coverage, and biovolume of attached cells, as well as to conduct more complicated evaluations of attachment, detachment, and cell growth on surfaces in real time (Caldwell and Germida, 1985). Enumeration of attached bacteria is often difficult when cells are arranged in clumps or aggregates, but even then, estimates can be made of the area covered by cells, and this can, within limits, be converted to biomass (Bjornsen, 1986). Some of the most extensive studies of bacterial attachment using image analysis have been conducted by D.E. Caldwell and J. Lawrence and co-workers (Caldwell and Germida, 1985; Caldwell and Lawrence, 1986; Lawrence and Caldwell, 1987; Lawrence et al., 1987). Although most of these studies have used flow cells, the same types of measurements are readily applicable to static systems.

Often attachment surfaces of interest are opaque, e.g. metals, minerals, anti-fouling coatings, macroorganisms, and incident light techniques must be used. Epifluorescent microscopy, utilizing fluorescent dyes, has been invaluable for assessing bacterial attachment to surfaces. Acridine orange and DAPI (4'6-diamidino-2-phenylindole) are the two most commonly used dyes, but Hoechst dyes have also been applied; all three dyes bind to nucleic acids in the cells. Paul (1982) compared acridine orange, DAPI, and Hoechst dyes for counting *Alteromonas citrea* and an *Aeromonas* sp. attached to glass or polystyrene and obtained similar counts with all three methods. Acridine orange is probably the most commonly used, but it tends to bind to some materials, including some plastics and detritus in samples from natural environments, resulting in background fluorescence.

Another fluorescent technique that is valuable not only for visualizing cells, but also for distinguishing among different organisms, is immunofluorescent labelling. Polyclonal or monoclonal antibodies can be generated from specific organisms or strains and congugated with a fluorochrome, such as fluorescein isothiocyanate (FITC). The bacteria on the attachment surface are then treated with the fluorescently tagged antibody, and the antibody should bind only with those organisms for which it is specific. Such techniques have been applied to environmental samples, by using antibodies generated from laboratory strains representing genera likely to be found in the

environment being studied (Dahle and Laake, 1982; Zambon et al, 1984). It is also possible to differentiate between two types of organism by using antibodies conjugated with distinctive fluorochromes, e.g., FITC and rhodamine.

Microscopy can be used not only to determine numbers, distribution, and identity of attached bacteria, but it can also be used to investigate the adhesive interaction itself by application of interference reflection microscopy (IRM) (Fletcher, 1988). With this technique, the bacterium is attached to the underside of a glass coverslip, and it is viewed by incident illumination that has been reflected from both the surface of the attached bacterium and the coverslip surface to which it is attached. The degree of interference between the reflected light from the two surfaces is a function of the distance separating the surfaces, and is detected as differences in image intensity. Thus, when the bacterium is at its closest approach to the surface, an image of maximum darkness is obtained. As the distance between cell and surface is increased, the image becomes progressively brighter, until an image of maximum brightness is obtained at a distance that is a function of the wavelength of light used and the refractive indices of the phases through which the light travels. For green light of 546 nm, this is approximately 100 nm. Therefore, the interaction between an attaching or attached cell and the glass substratum (in terms of separation distance) can be evaluated with living organisms in real time. The influence of environmental factors, e.g., electrolyte concentration and nutrients, on the interaction can also be tested. For example, in experiments with two strains of *P. fluorescens*, changes in image intensity consistent with a decrease in separation distance were induced by the addition of sodium chloride, calcium chloride, or lanthanum chloride (Fletcher, 1988). Furthermore, in some cases, these changes were reversible upon the replacement of electrolyte with distilled water. From the results, some inferences may be made concerning the significance of electrostatic interactions and the influence of electrolytes on the adhesion of these organisms.

Electron microscopy can also be used to evaluate numbers and distribution of attached bacteria, as well as provide information on attachment structures such as fimbriae, flagella, or polymeric adhesives. Scanning electron microscopy (SEM) is particularly useful, as it can be applied to a variety of substrata, such as metals, minerals, and plant and animal surfaces. A potential disadvantage of SEM is that preparation techniques can denature polymers through dehydration, and artefacts are an inevitable result. Polymeric material that is highly hydrated in living biofilms will be condensed and resemble fibrils after conventional chemical and fixation methods followed by critical point drying or argon replacement-induced drying (Lamb and Ingram, 1979). However, such condensates may be reduced by cryofixation (Fraser and Gilmour, 1986).

Transmission electron microscopy has been used to visualize attached cells, either through the used of negative-stained or shadow preparations or thin sections of specimens (Marshall, 1971a). Negative staining is similar in principle to light microscopy or SEM in that the attached bacterium is viewed from above, rather than edge-on. The resolution is greater than that achieved with SEM, so that adhesive polymers and attachment structures, e.g., pili, fimbriae, can be observed. Also, like SEM, negative staining and TEM allows determination of numbers and distribution of

bacteria. However, a disadvantage is that the substratum is restricted to coated electron microscope grids.

To examine the interface between an attached bacterium or biofilm and the substratum, thin sections of material can be prepared. Moreover, extracellular polymers can be stained with polyanionic stains, e.g. ruthenium red (Fletcher and Floodgate, 1973; Costerton, 1980). Substrata that can be sectioned are required for vertical sections, and prepolymerized resin (Marshall and Cruickshank, 1973) and cellulose ester membrane filters (Fletcher and Floodgate, 1973) have been used as surfaces for attachment.

The recent development of scanning confocal laser microscopy (SCLM) has considerably broadened our horizons with respect to microscopical examination of biofilms, and should revolutionize our approach to the study of multicellular accumulations of attached cells. With SCLM, a conical illuminating beam of light that is focused to a point by an objective lens is scanned through the sample. A light detector, such as a photomultiplier tube, then measures the interaction of the illuminating probe with each point in the sample. The light measured can be reflected light or fluorescence emitted in response to the probe. Fluorescein has been used to visualize cells in biofilms, as the fluorochrome was dissolved in the medium and excluded by the cells (Lawrence et al., 1991). The image produced by the scanning detector can then be displayed on a video monitor and digitized into a computer framestore (Shotton and White, 1989). The result is an image that is free of the out-of-focus blur characteristic of light microscopy. Furthermore, because the probe can be used to scan at various depths throughout the specimen, it is possible to construct three-dimensional images, or to produce "optical sections". SCLM has been used to visualize noninvasively hydrated biofilms of *P. aeruginosa*, *P. fluorescens*, and *Vibrio parahaemolyticus*, and variations in cell density and arrangement were documented (Lawrence et al., 1991).

3.2. MEASUREMENT OF ATTACHED CELL BIOMASS

3.2.1. *Introduction.* Despite all its advantages, microscopy has its limitations for assessing bacterial attachment to surfaces. First, it is restricted to relatively flat surfaces, and cannot be applied to porous or topographically complex materials. Second, apart from histochemical data, it provides no information on the chemical composition of attached populations. Finally, microscopy is probably more susceptible to "sampling error" than some other methods, as a comparatively small area must be examined. Thus, for architecturally complex substrata or those with large surface areas, it can be preferable to evaluate attachment by measuring the amount of a chemical biomarker, which is an indicator of biomass. Alternatively, numbers of cells may be determined by removing them quantitatively from the surface and determining their numbers by culturing techniques.

3.2.2. *Determination of Numbers.* Numbers of attached bacteria have frequently been determined by removal of cells, followed by their enumeration by total or viable counts. There is a serious deficiency with this approach, however, in that it is extremely difficult to remove attached cells quantitatively. The strength of adhesion, and thus the resistance

to removal, varies depending upon the organisms, the surfaces, the length of time the cells have been attached, and the type and amount of associated inorganic deposits and organic adhesives. A number of methods have been applied to remove attached bacteria, ranging from relatively ineffective swabbing, to treatment with ion sequestering agents (e.g., pyrophosphate) or detergents, to sonication and vortexing. Comparisons of such techniques have been made when removing bacteria from sediment particles (Litchfield et al., 1975; McDaniel and Capone, 1985). It is impossible to recommend any one procedure, and optimum extraction procedures should be determined in individual cases. If bacterial numbers are to be determined by plating on solid media, then maintenance of viability must be considered when devising methods for maximum removal of cells.

A semi-quantitative estimate of bacterial numbers on transparent surfaces can be rapidly obtained by staining the attached cells with crystal violet, or a similar stain, and then measuring the absorption of the stained cells at the appropriate wavelength in a spectrophotometer. The absorbance can then be calibrated to numbers of cells, which has been determined separately on a standard series of attached cell densities by microscopy.

3.2.3. *Chemical Biomarkers.* Various structural components of the cell, e.g. protein, carbohydrate, ATP, DNA, lipopolysaccharide (LPS), can be used to determine the amount of cell biomass. The amount of such a biomarker is determined and then related to biomass using a separately determined calibration. The effectiveness of such methods depends upon the sensitivity and precision of the detection and quantitation techniques, but they are extremely valuable when biomass is sufficient. The proportions of particular components may vary with different organisms or nutritional status, which may make it possible to assess community members or their metabolic state.

The biomass of attached bacteria may be indirectly assessed by extraction of ATP from cells and determination of its concentration by the luciferin/luciferase reaction (Harber et al., 1983). Similarly, numbers of attached cells have been evaluated by measuring their DNA content with a fluorometric technique using Hoechst staining (Paul and Loeb, 1983). If the attached bacteria are Gram-negative, their biomass can be evaluated by determination of LPS concentration by using *Limulus* amoebocyte lysate (Dexter et al., 1975). This extract from *L. polyphemus* (horseshoe crab) forms a gel or turbid solution in the presence of the protein-LPS endotoxin of Gram-negative bacteria, and the optical density of the solution is related to bacterial density. Other cell constituents that have been measured to assess cell biomass include teichoic acids in Gram-positive bacteria (Gehron et al., 1984) and muramic acid (Findlay et al., 1983).

Extensive studies of bacterial biofilms and their nutritional status have been carried out using a biomarker approach. D.C. White and co-workers (White, 1984; McKinley et al., 1988) have measured cell constituents, including phospholipid fatty acids (Gehron and White, 1983) and lipid A of lipopolysaccharides (Parker et al., 1982). Recently, sensitivity of phospholipid fatty acid analysis has been increased markedly and applied to detect differences between free cells and attached biofilms of *P. atlantica* (Tunlid et al., 1989).

4. Radiotracer Labelling

Numbers of attached bacteria can be indirectly assessed by first labelling the organisms with a radiotracer, allowing them to attach, and then measuring by scintillation counting the amount of radioactivity associated with the surface. This is a relatively rapid approach, and it is precise and sensitive. The disadvantage is that preincubation of the cells with the labelled substrate may influence their attachment properties. Supplementary experiments are required to calibrate scintillation counts to cell biomass, confirm that the radiolabel is stably retained within the cells for the required period of time, and ensure that radiation is not absorbed by test surfaces resulting in erroneously low counts. Two substrates that have commonly been used to label cells are leucine (Pringle and Fletcher, 1983) and thymidine (Ørstavik, 1977).

5. Genetic Techniques

Recent advances in molecular biology are resulting in methods that may allow *in situ* detection and enumeration of specific organisms on surfaces. Nucleic acid probes, which hybridize to cellular DNA or RNA, have been applied successfully to environmental isolates (Sayler et al., 1985; Holben et al., 1988). Considerable progress has been made recently in the development of oligonucleotide probes for detection of 16S rRNA sequences that are specific for particular species or genera of microorganisms (Stahl et al., 1988). Giovannoni et al. (1988) used oligodeoxynucleotide probes complementary to 16S rRNA to characterize bacteria that had been artificially attached to a glass slide by first dipping the slide in gelatin and applying the bacteria to the gelatin-coated slide. Hybridization to individual cells was then visualized by microautoradiography. More recently, five fluorescently tagged 16S rRNA probes were used to visualize different sulfate-reducing bacteria within developing and mature biofilms (Amann et al., 1992). Such techniques offer considerable promise for future understanding of colonization of biofilms and interactions among different taxonomic and functional groups, particularly when they may be combined with confocal laser scanning microscopy.

6. Conclusions

There are many types of adhesion assay available to researchers, and more are becoming available with developments in confocal microscopy and genetic and biochemical techniques. However, the more that we investigate bacterial adhesion to surfaces the more apparent is the enormous variability and diversity in bacterial attachment mechanisms and behaviors. The degree and nature of bacterial attachment in a given situation is highly dependent upon the organisms, substrata, and environmental conditions, and it is rarely possible to extrapolate from one system to another. Nevertheless, as new methods are developed and old ones refined, it may become easier to select methods that are well-suited for the attachment system being investigated. It will always be extremely important when selecting methods to bear in mind the specific

questions being addressed and the appropriate conditions for finding answers to those questions.

7. References

Allison, D.G. and Sutherland, I.W. (1984) 'A staining technique for attached bacteria and its correlation to extracellular carbohydrate production', J. Microbiol. Meth. 2, 93-99.

Amann, R.I., Stromley, J., Devereux, R., Key, R., and Stahl, D.A. (1992) 'Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms', Appl. Environ. Microbiol. 58, 614-623.

Baker, J.H. (1984) 'Factors affecting the bacterial colonization of various surfaces in a river', Can. J. Microbiol. 30, 511-515.

Bartlett, D.H., Wright, M.E. and Silverman, M. (1988) 'Variable expression of extracellular polysaccharide in the marine bacterium *Pseudomonas atlantica* is controlled by genome arrangement', Proc. Natl. Acad. Sci. USA 85, 3923-3927.

Bjornsen, P.K. (1986) 'Automatic determination of bacterioplankton biomass by image analysis', Appl. Environ. Microbiol. 51, 1199-1204.

Busscher, H.J., Uyen, M.H.W.J.C., van Pelt, A.W.J., Weerkamp. A.H. and Arends, J. (1986a) 'Kinetics of adhesion of the oral bacterium *Streptococcus sanguis* CH3 to polymers with different surface free energies', Appl. Environ. Microbiol. 51, 910-914.

Busscher, H.J., Uyen, M.H.W.J.C., Weerkamp, A.H., Postma, W.J. and Arends, J. (1986b) 'Reversibility of adhesion of oral streptococci to solids', FEMS Microbiol. Lett. 35, 303-306.

Caldwell, D.E. and Germida, J.J. (1985) 'Evaluation of difference imagery for visualizing and quantitating microbial growth', Can. J. Microbiol. 31, 35-44.

Caldwell, D.E. and Lawrence, J.R. (1986) 'Growth kinetics of *Pseudomonas fluorescens* microcolonies within the hydrodynamic boundary layers of surface microenvironments', Microb. Ecol. 12, 299-312.

Costerton, J.W. (1980) 'Some techniques involved in study of adsorption of microorganisms to surfaces', in G. Bitton and K.C. Marshall, (eds.), Adsorption of Microorganisms to Surfaces, John Wiley, New York, pp. 403-423.

Dahle, A.B. and Laake, M. (1982) 'Diversity dynamics of marine bacteria studied by immunofluorescent staining on membrane filters', Appl. Environ. Microbiol. 43, 169-176.

Dexter, S.C., Sullivan, J.D.Jr., Williams, J. III and Watson, S.W. (1975) 'Influence of substrate wettability on the attachment of marine bacteria to various surfaces', Appl. Microbiol. 30, 298-308.

Feldner, J., Bredt, W. and Kahane, I. (1983) 'Influence of cell shape and surface charge on attachment of *Mycoplasma pneumoniae* to glass surfaces', J. Bacteriol. 153, 1-5.

Findlay, R.H., Moriarty, D.J.W. and White, D.C. (1983) 'Improved method of determining muramic acid from environmental samples', Geomicrob. J. 3, 133-150.

Fletcher, M. (1976) 'The effects of proteins on bacterial attachment to polystyrene', J. Gen. Microbiol. 94, 400-404.

Fletcher, M. (1977) 'The effects of culture concentration and age, time and temperature on bacterial attachment to polystyrene', Can. J. Microbiol. 23, 1-6.

Fletcher, M. (1988) 'Attachment of *Pseudomonas fluorescens* to glass and influence of electrolytes on bacterium-substratum separation distance', J. Bacteriol. 170, 2027-2030.

Fletcher, M. and Floodgate, G.D. (1973) 'An electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces', J. Gen. Microbiol. 74, 325-334.

Fletcher, M. and Loeb, G.I. (1979) 'The influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces', Appl. Environ. Microbiol. 37, 67-72.

Fraser, T.W. and Gilmour, A. (1986) 'Scanning electron microscopy preparation methods: their influence on the morphology and fibril formation in *Pseudomonas fragi* (ATCC 4973)', J. Appl. Bacteriol. 60, 527-533.

Gehron, M.J. and White, D.C. (1983) 'Sensitive assay of phospholipid glycerol in environmental samples', J. Microbiol. Meth. 1, 23-32.

Gehron, M.J., Davis, J.D., Smith, G.A. and White, D.C. (1984) 'Determination of the Gram-positive content of soils and sediments by analysis of teichoic acid components', J. Microbiol. Meth. 2, 165-176.

Giovannoni, S.J., DeLong, E.F., Olsen,G.J. and Pace, N.R. (1988) 'Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells', J. Bacteriol. 170, 720-726.

Gordon, A.S. (1987) 'Influence of alginate on attachment of *Vibrio* spp. to stainless steel surfaces in seawater', Appl. Environ. Microbiol. 53, 1175-1177.

Gordon, A.S., Gerchakov, S.M. and Udey, L.R. (1981) 'The effect of polarization on the attachment of marine bacteria to copper and platinum surfaces', Can. J. Microbiol. 27, 698-703.

Harber, M.J., Mackenzie, R. and Asscher, A.W. (1983) 'A rapid bioluminescence method for quantifying bacterial adhesion to polystyrene', J. Gen. Microbiol. 129, 621-632.

Hogt, A.H., Dankert, J., Vries, J.A.de and Feijen, J. (1983) 'Adhesion of coagulase-negative staphylococci to biomaterials', J. Gen. Microbiol. 129, 2959-2968.

Holben, W.E., Jansson, J.K., Chelm, B.K. and Tiedje, J.M. (1988) 'DNA probe method for the detection of specific microorganisms in the soil bacterial community', Appl. Environ. Microbiol. 54, 703-711.

Isaacson, R.E. (1985) 'Pilus adhesins', in D.C. Savage and M. Fletcher (eds.), Bacterial Adhesion, Plenum Press, New York, pp. 307-336.

Knox, K.W., Hardy, L.N. , Markevics, L.J., Evans, J.D. and Wicken, A.J. (1985) 'Comparative studies on the effect of growth conditions on adhesion, hydrophobicity, and extracellular protein profile of *Streptococcus sanguis* G9B', Infect. Immun. 50, 545-554.

Lamb, J.C. and Ingram, P. (1979) 'Drying of biological specimens for scanning electron microscopy directly from ethanol', Scanning Elec. Microsc. 3, 459-464.

Lawrence, J.R. and Caldwell, D.E. (1987) 'Behavior of bacterial stream populations within the hydrodynamic boundary layers of surface microenvironments', Microb. Ecol. 24, 15-27.

Lawrence, J.R., Delaquis, P.J., Korber, D.R. and Caldwell, D.E. (1987) 'Behavior of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments', Microb. Ecol. 14, 1-14.

Lawrence, J.R., Korber, D.R., Hoyle, B.D., Costerton, J.W., and Caldwell, D.E. (1991) 'Optical sectioning of microbial biofilms', J. Bacteriol. 173, 6558-6567.

Litchfield, C.D., Rake, J.B., Zindulis, J., Watanabe, R.T. and Stein, D.J. (1975) 'Optimization of procedures for the recovery of heterotrophic bacteria from marine sediments', Microb. Ecol. 1, 219-233.

Ludwicka, A., Jansen, B., Wadstrom, T. and Pulverer, G. (1984) 'Attachment of staphylococci to various synthetic polymers', Abl. Bakt. Hyg. A 256, 479-489.

Marshall, K.C. and Cruickshank, R.H. (1973) 'Cell surface hydrophobicity and the orientation of certain bacteria at interfaces', Arch. Microbiol. 91, 29-40.

Marshall, K.C., Stout, R. and Mitchell, R. (1971a) 'Selective sorption of bacteria from seawater', Can. J. Microbiol. 17, 1413-1416.

Marshall, K.C., Stout, R. and Mitchell, R. (1971b) 'Mechanism of the initial events in the sorption of marine bacteria to surfaces', J. Gen. Microbiol. 68, 337-348.

McDaniel, J.A. and Capone, D.G. (1985) 'A comparison of procedures for the separation of aquatic bacteria from sediments for subsequent direct enumeration', J. Microbiol. Meth. 3, 291-302.

McEldowney, S. and Fletcher, M. (1986) 'Effect of growth conditions and surface characteristics of aquatic bacteria on their attachment to solid surfaces', J. Gen. Microbiol. 132, 513-523.

McEldowney, S. and Fletcher, M. (1987) 'Adhesion of bacteria from mixed cell suspension to solid surfaces', Arch. Microbiol. 148, 57-62.

McKinley, V.L., Costerton, J.W. and White, D.C. (1988) 'Microbial biomass, activity, and community structure of water and particulates retrieved by backflow from a waterflood injection well', Appl. Environ. Microbiol. 54, 1383-1393.

Molin, G., Nilsson, I. and Stenson-Holst, L. (1982) 'Biofilm build-up of *Pseudomonas putida* in a chemostat at different dilution rates', Eur. J. Appl. Microbiol. Biotechnol. 15, 218-222.

Ørstavik, D. (1977) 'Sorption of *Streptococcus faecium* to glass', Acta Path. Microbiol. Scand. Sect. B 85, 38-46.

Parker, J.H., Smith, G.A., Fredrickson, H.L., Vestal, J.R. and White, D.C. (1982) 'Sensitive assay, based on hydroxy-fatty acids from lipopolysaccharide lipid A for gram negative bacteria in sediments', Appl. Environ. Microbiol. 44, 1170-1177.

Paul, J.H. (1982) 'Use of Hoechst dyes 33258 and 33342 for enumeration of attached and planktonic bacteria', Appl. Environ. Microbiol. 43, 939-944.

Paul, J.H. and Loeb, G.I. (1983) 'Improved microfouling assay employing a DNA-specific fluorochrome and polystyrene as substratum', Appl. Environ. Microbiol. 46, 338-343.

Pringle, J.H. and Fletcher, M. (1983) 'Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces', Appl. Environ. Microbiol. 45, 811-817.

Pringle, J.H., Fletcher, M. and Ellwood, D.C. (1983) 'Selection of attachment mutants during the continuous culture of *Pseudomonas fluorescens* and relationship between attachment ability and surface composition', J. Gen. Microbiol. 129, 2557-2569.

Rosenberg, M. and Rosenberg, E. (1985) 'Bacterial adherence at the hydrocarbon-water interface', Oil Petrochem. Poll. 2, 155-162.

Satou, N., Satou, J., Shintani, H. and Okuda, K. (1988) 'Adherence of streptococci to surface-modified glass', J. Gen. Microbiol. 134, 1299-1305.

Sayler, G.S., Shields, M.S., Tedford, E.T., Breen, A., Hooper, S.W., Sirotkin, K.M. and Davis, J.W. (1985) 'Application of DNA-DNA colony hybridization to the detection of catabolic genotypes in environmental samples', Appl. Environ. Microbiol. 49, 1295-1303.

Shotton, D. and White, N. (1989) 'Confocal scanning microscopy: three dimensional biological imaging', TIBS 14 , 435-439.

Stahl, D.A., Flesher, B., Mansfield, H.R. and Montgomery, L. (1988) 'Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology', Appl. Environ. Microbiol. 54, 1079-1084.

Tunlid, A., Ringelberg, D., Phelps, T.J., Low, C., and White, D.C. (1989) 'Measurement of phospholipid fatty acids at picomolar concentrations in biofilms and deep subsurface sediments using gas chromatography and chemical ionization mass spectrometry", J. Microbiol. Meth. 10, 139-153.

van Pelt, A.W.J., Weerkamp, A.H., Uyen, M.H.W.J.C., Busscher, H.J., de Jong, H.P. and Arends, J. (1985) 'Adhesion of *Streptococcus sanguis* CH3 to polymers with different surface free energies', Appl. Environ. Microbiol. 49, 1270-1275.

Weerkamp, A.H., Handley, P.S., Baars, A. and Slot, J.W. (1986) 'Negative staining and immunoelectron microscopy of adhesion-deficient mutants of *Streptococcus salivarius* reveal that the adhesive protein antigens are separate classes of cell surface fibril', J. Bacteriol. 165, 746-755.

Weerkamp, A.H., van der Mei, H.C. and Slot, J.W. (1987) 'Relationship of cell surface morphology and composition of *Streptococcus salivarius* K^+ to adherence and hydrophobicity', Infect. Immun. 55, 438-445.

White, D.C. (1984) 'Chemical characterization of films', in K.C. Marshall (ed.), Microbial Adhesion and Aggregation, Springer-Verlag, Berlin, pp. 159-176.

Zambon, J.J., Huber, P.S., Meyer, A.E., Slots, J., Fornalik, M.S. and Baier, R.E. (1984) '*In situ* identification of bacterial species in marine microfouling films by using an immunofluorescence technique', Appl. Environ. Microbiol. 48, 1214-1220.

BIOFILM LABORATORY METHODS: THE USE OF FLOW REACTORS

JAMES D. BRYERS
THE CENTER FOR
BIOCHEMICAL ENGINEERING
DUKE UNIVERSITY
DURHAM, NORTH CAROLINA
27706 USA

WILLIAM G. CHARACKLIS
THE CENTER FOR INTERFACIAL
MICROBIAL PROCESS ENGINEERING
MONTANA STATE UNIVERSITY
BOZEMAN, MONTANA
59717 USA

1. Introduction

"Begin at the beginning," the King said gravely, "and go on till you come to the end; then stop."
Lewis G. Carroll, Alice Through the Looking Glass

Investigations of biofilm formation and persistence require a fundamental approach in the collection and analysis of data, irrespective of the specific environment or application. This approach must be based upon the principles of process analysis (see Bryers and Characklis, this volume) and the concomitant need to provide comprehensive and compatible data with which to evaluate appropriate mass balance equations. Experimental reactor systems employed in either *(a)* controlled laboratory environments, *(b)* field applications, or *(c) in vivo* biomedical situations should be designed and operated in order provide the appropriate data under known conditions to facilitate this mass balance approach.

1.1 REACTOR ENGINEERING FUNDAMENTALS

To define it rudely but not inaptly, engineering is the art of doing that well with one dollar which any bungler can do with two dollars.
Arthur M. Wellington

Reactor engineering describes three types of ideal reactor concepts: the continuously-fed stirred tank reactor (CSTR), the batch reactor, and the plug flow reactor (PFR). *Batch* reactors essentially represent a completely closed reaction volume that contains all the necessary reactants under the conditions necessary to carry out the desired reaction. There is no input or outlet from a batch system, consequently reactant and product concentrations will change with time. Sufficient mixing of the reaction volume is assumed so that there are no spatial gradients in reaction mixture temperature or density - *i.e.*, the reaction volume is well-mixed.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 615–629.

The *CSTR* is essentially a constant volume open system that receives a continuous supply of the necessary reactants while providing a completely mixed reaction volume. After a certain time period under the constant delivery of reactants and withdrawal of reactor contents, the system attains a steady-state in the various reaction components. The advantages of the CSTR is that, at steady-state, the bulk liquid phase is uniform in composition which makes sampling and reaction analysis simple and reproducible.

While CSTRs and batch reactors represent the ideal in a well mixed, gradient-less reaction system, *plug flow* reactors are the other extreme in that elements or packets of fluid are assumed to move through the reaction volume without exchanging or mixing of material between adjacent elements. Reactants are fed at a continuous, constant rate to the reactor thus establishing, at any position between the inlet and outlet, a steady-state in composition. Thus while reactant and product concentrations may change with location as an element proceeds through the reactor, at any one location the concentrations are constant with time.

Table 1 summarizes continuity equations (mass balances) for a single component A, a reactant in the reaction $A \rightarrow B$, that conforms the the reaction rate expression $r_A = \upsilon_A r = \upsilon_A f(A)$, for the three basic ideal reactor types.

Many biofilm systems of industrial application and ecological concern occur in systems that can be classified as one of these reactor types. We shall provide examples of these systems in the appropriate sections below. Realize that in laboratory reactors or field surveillance systems, the specific biofilm reactor design selected can be operated in either of these three ideal modes. Also realize that while one can attempt to affect ideal reactor behavior in an experimental system, the degree of ideality must be assessed under the operating conditions in question.

Any mental activity is easy if
it does not need to take reality
into account.
Proust

2. Biofilm Reactor Systems Prerequisites

2.1 SYSTEM COMPONENTS

Irrespective of the application or study objective, a biofilm experimental system must contain certain critical components: *(a)* the biofilm study reactor itself (where the surface accumulation of cells and biofilm will be studied) and *(b)* the environmental support system which controls the experimental conditions (*e.g.*, temperature, flow velocity, nutrient concentrations, pH). This chapter will emphasize only those experimental systems that create a flow of the bulk fluid phase; this flow can be affected by either the study reactor itself or the environmental support system. Flow of the bulk liquid in a biofilm experiment can be mandated for several overlapping reasons: to study biofilm accumulation either *(a)* as a function of fluid velocity or shear stress, *(b)* as a function of a nutritional limitation or kinetic parameter (nutrients or growth rate) in a simulated flow environment, or *(c)* as a means of eliminating any potential mass transfer rate limitations. In field situations, the reactor may rely on the natural ecosystem or an operating engineered system to dictate nutritional and hydrodynamic

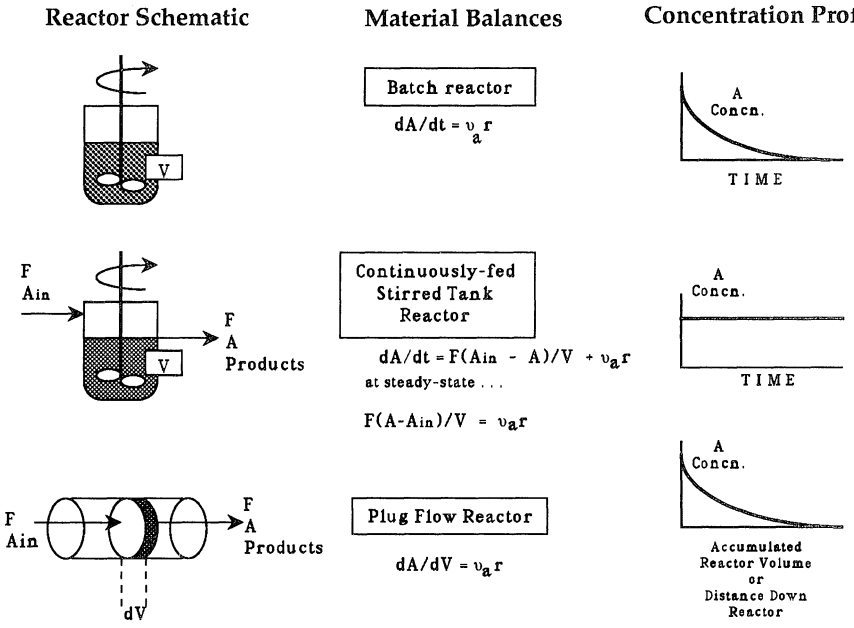


Table 1. Summary of ideal reactor concepts and design equations.

conditions. Our emphasis here on flow systems is complemented by a similar chapter in this volume on static or quiescent systems by M. Fletcher.

The experimental laboratory system operating *in vitro* should have the ability to control or vary a number of experimental parameters as summarized in Table 2. Control of experimental parameters is the luxury of *in vitro* laboratory systems. While certain parameters can not be controlled

TABLE 2. Experimental Variables

Chemical Parameters	*Physical Parameters*	*Biological Parameters*
substrate type	temperature	microorganism type
substrate concentration	fluid shear stress	culture type (mixed vs. pure)
pH	heat flux	suspended cell concn.
inorganic ions	surface composition	antagonist organisms
dissolved oxygen	surface texture	
microbial inhibitors	fluid residence time	

in field studies or *in vivo* situations, the reactor system should at least provide for their determination.

2.2 DIAGNOSTIC METHODS

Specific analyses of cell adhesion, biofilm formation, and reactivity will not be addressed here in great detail but are summarized elsewhere (Characklis and Marshall, 1990); specific diagnostic techniques for quantifying attached cell quantity and reactivity, biofilm amount, composition, and various properties are addressed in greater detail in this volume. Here we emphasize the various techniques employed in the study of biofilms and specifically detail how a detection method influences the design and construction of the study reactor.

2.2.1 *Invasive Sampling.* Detection of biofilms often requires direct sampling and removal of a finite quantity of attached material from a reactor surface for a number of destructive analytical procedures that measure overall biofilm amount (mass or thickness), a component of the biofilm structure (biofilm carbon, cell number, biofilm protein), or biofilm cell activity (ATP, dehydrogenase activity, active DNA synthesis). Detection of parameters in the biofilm as determined by sensor probes (pH, DO, temperature, specific ions) still requires the invasive penetration of the biofilm by the probe, under typically quiescent conditions, although Characklis and co-workers have recently documented probe use in flowing fluid systems. Such reactor requirements allow for either *(a)* ease of access to the interior of the reactor in order to physically scrape off biofilm from a known sample area, or *(b)* the ability to aseptically remove a representative portion of the substratum and (hopefully) its portion of the biofilm, or *(c)* provide access to the biofilm for the penetration of diagnostic sensor probes. Reactor designs to provide access for sampling involve removable sections of substratum; some of which are quite arcane, other rather ingenious. All destructive sampling devices, however,

have an impact on the construction of the reactor. Examples of three different removable sample techniques are presented in Figure 1.

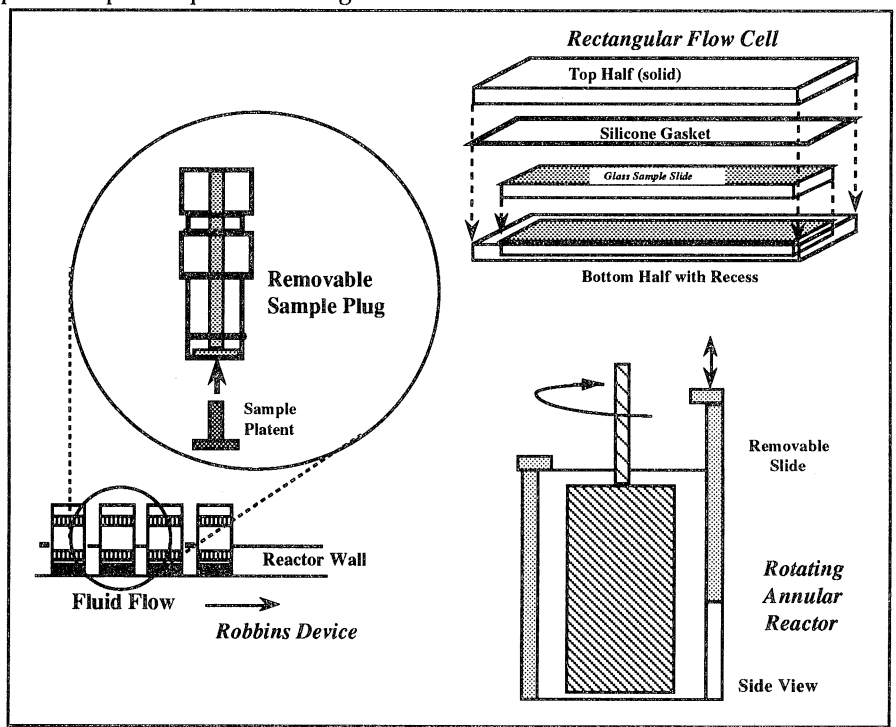


Figure 1. Examples of different types of removable reactor test surfaces

2.2.2. *Non-invasive Diagnostics.* All biofilm researchers would ideally like the analysis of the immobilized cell layer to create as little disturbance to the biofilm and the reactor operation as possible. Regrettably, most parameters of interest in biofilm accumulation require destructive sampling and analyses.

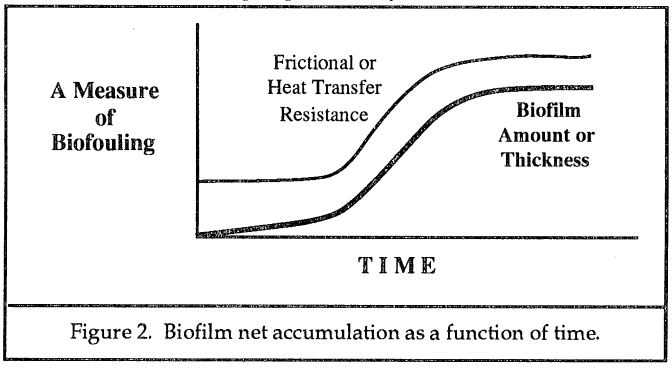


Figure 2. Biofilm net accumulation as a function of time.

One class of non-invasive techniques does not measure the biofilm itself but rather monitors the effects of biofilm formation on certain system performance parameters. For instance, biofilm formation can create significant increases in fluid frictional and heat transfer resistances (Picologlou *et al.*, 1980; Turakhia and Characklis, 1983); increases that respond in time in a fashion similar to biofilm accumulation (Figure 2). Consequently, the

measure of increases in frictional resistance (increased pressure drop at constant fluid velocity, decreased fluid velocity at constant pressure drop, or increased torque at a constant rotational speed in a RAR) can be non-invasively monitored continuously throughout biofilm accumulation by way of on-line flow meters, pressure transducers, or torque monitors. On-line determination of heat transfer resistances requires detection of bulk liquid temperature and substratum surface temperature and heat flux. Siebel *et al.* (1991) reports a non-invasive light absorbance technique that is based on the absorbance of infrared light by the biofilm; it requires a light emitter positioned on one side of the biofilm reactor and a detector on the other, and a transparent biofilm reactor. Differences in the current are presently being correlated to biofilm amount.

Another class of non-invasive detection methods are measures of system performance such as temperature, pH, dissolved oxygen, specific ion probes where the detection probe is built directly into the reactor, flush with the surface of the target substratum. Consequently, one can derive continual on-line measurement of certain parameters (*e.g.*, pH, temperature, dissolved oxygen) at the base of the biofilm with elapsed time. The disadvantage of surface mounted probes is that, once in place, any drift in signal can not be easily corrected.

True non-invasive diagnostics of biofilm accumulation, amount, and biofilm-cell reactivity are limited to relatively few sophisticated techniques. The simplest of these techniques is on-line video microscopy that places the biofilm reactor within the viewing field of a microscope. This technique requires a reactor dimension compatible with the microscope, preferably flat reactor surface on which to focus, and obviously a reactor that is transparent. Microscopic observation can be quantified and preserved by use of video recorders or image analyzer systems which digitize observed images on a "degree of grayness" basis and saves these images on a computer for future numerical interpretations (Figure 3). Examples of such systems applied to cell adhesion are described by Escher, (1986) and Feurerstein & Kush (1986).

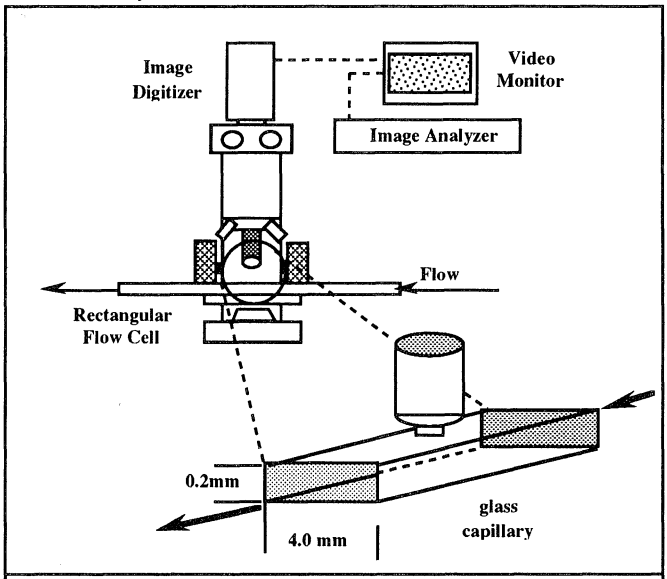


Figure 3. Illustration of an on-line video microscope system for continuous cell adhesion and biofilm surveillance.

A second technique that has tremendous potential for providing non-invasively information on the molecular chemistry of cell adhesion is Attenuated Total Reflectance (ATR) waveguides integrated within flow cells coupled with Fourier Transform Infrared Spectroscopy (FT-IR). Atoms and groups of atoms within a molecule vibrate with a characteristic frequency and will absorb light at these frequencies. Light containing infra-red frequencies can be focused on a molecule and the amount of light absorbed measured as a function of frequency. Specific IR absorptions can thus be assigned to particular bonds and alterations in these bonds due to changes in local environment can be assessed from resultant spectral details. Recent increases in the capability of FT-IRs and the focusing of the IR wave within specific crystal wave guides allows one to establish a standing IR wave at the surface of the crystal. Fourier transform signal processing and multiple scanning allows aqueous samples to be processed where the IR spectra of molecules directly adjacent to the waveguide can be collected. In biological systems the IR absorbance of water must be subtracted via computer manipulations to provide the IR spectra of molecules accumulating at the crystal surface. The effective depth of penetration for the evanescent IR wave is a function of crystal material and the wavenumber but ranges about 0.3 - 0.7 μm thus providing spectra that reflect those chemical species directly adjacent to the surface. Bremer and Geesey (1991) report the chemical changes that occur, after inoculating with a microbial culture, at a germanium ATR crystal situated within a flow cell as detected by FT-IR. Spectral intensities representing various bonds within proteins and polysaccharide are seen to accumulate with time indicating the feasibility of the ATR/FT-IR system to detect biofilm formation; unfortunately, no independent assessment of biofilm accumulation was made. Since the IR wave can only penetrate less than 1 μm into the biofilm, the reported increases in absorbance intensities most likely indicate increases in cell surface coverage of the crystal and not increases in biofilm thickness.

3. Biofilm Reactor Designs

3.1 CHEMOSTAT VESSELS

A large number of biofilm studies have been carried out in laboratory suspended culture bioreactor vessels where biofilm forms, uncontrolled, on all surfaces exposed to the aqueous phase. Such studies typically adapt standard lab fermenter vessels to accommodate for periodic sampling of the biofilm. The simplest method is to introduce, by way of an exist sterile sampling port, a glass rod through the vessel head plate and suspended to a known depth (thus a known exposed surface area) in the culture fluid. With such a system, Molin & Nilsson (1983) followed the accumulation of a *Pseudomonas putida* ATCC 11172 biofilm within a chemostat through progressive increases in system dilution rate. Unfortunately, the many surfaces in a fermentation vessel in contact with fluid are highly irregular and are exposed to locally different hydrodynamic forces such that resultant biofilms can differ spatially in thickness and cell concentration. Thus, one can estimate from such a removable glass rod, only an average biofilm amount throughout the vessel. In addition, the number of available sample ports in a standard fermenter head plate is finite, providing limited surface area for biofilm samples. All these limitations greatly reduce the practicality a classical fermentation vessel for quantitative analyses of biofilm formation processes.

3.2 ROTATING ANNULAR REACTORS

One advantage of the continuously fed bioreactor (*i.e.*, chemostat) above is that the entire culture is supposedly at the same growth rate, exposed to the same growth limiting substrate

concentration. But as a biofilm study reactor, a fermentation vessel's surface area:volume ratio is poor and the prevailing hydrodynamics in and around the numerous submerged sensing devices and baffles are, at best, ill-defined. Consequently, the Rotating Annular Reactor (RAR) was developed (Kornegay, 1967) to alleviate the ills of a fermenter while retaining control over certain growth rate parameters. The same reactor coupled to an on-line torque monitor has been termed a RotoTorque reactor by its developers (NSF-Engineering Research Center).

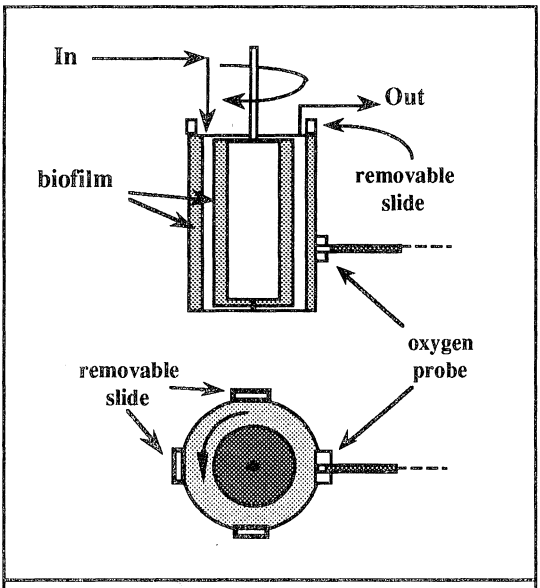


Figure 4. Rotating annular reactor.

An example of this type of RAR is illustrated in Figure 4. A RAR comprises two concentric cylinders, fitted one within another separated by a small gap. One cylinder remains stationary and the other is free to rotate. The most common design has the outer cylinder stationary while the inner cylinder is rotated mechanically at a rotational speed to affect a desired surface velocity or shear stress. At extremely high rotational speeds, this particular design of the RAR can lead to Taylor vortices; a situation of non-ideal mixing where layers of rings of fluid vortices are formed where fluid moving to the outer cylinder wall curls and reverses direction returning towards the inner cylinder (Schlichting, 1968). The alternative version of a rotating outer cylinder (LaMotta, 1974) and stationary inner cylinder provides more hydrodynamic stability but the outer cylinder proves more difficult to mechanically rotate and does not allow easy access for surface sampling. Another method to break up Taylor vortices is by constructing the rotating inner cylinder with draft tubes to internally recirculate fluid.

Removable slides or test plugs as illustrated in Figure 1 can be constructed within the outer, stationary cylinder of a RAR to provide for destructive sampling of the developing biofilm. If the gap width between inner and outer cylinder is small, then the RAR design provides a bioreactor system that has a very high surface area: volume ratio which promotes biofilm development while minimizing suspended growth. Further, if the system is operated at a residence time less than the maximum generation time of the culture, then planktonic growth is negligible and all microbial activity within the RAR can be attributed to the biofilm. Most of the surface area in a RAR is exposed to the same uniform shear stresses providing ample sampling area of a relatively uniform biofilm. One advantage of the RAR has over flow cell reactors (next section) is that shear stress and linear velocity are determined by rotational speed and are thus independent of the nutrient input flow rate to the system. A torque transducer can be mounted on the shaft between the motor and the rotating cylinder to monitor continuously the drag force on the moving cylinder. Frictional resistance increases due to biofilm formation can thus be calculated from rotational speed and torque measurements. The Reynolds number for a RAR, based on the rotational or tangential velocity component, can be written,

$$Re_R = \Omega\alpha R_o^2\rho/\eta \quad (1)$$

where Ω is the rotational speed, α is the ratio of the radii of the inner to outer cylinder, R_o is the inner radius of the outer cylinder, ρ and η are the density and absolute viscosity of the fluid, respectively. Another analogous dimensionless group often used with the RAR geometry is the Taylor number (Taylor, 1923):

$$Ta = v_{in}\, R_o(1-\alpha)^{1.5}/\upsilon(\alpha)^{0.5} \quad (2)$$

where v_{in} is the linear velocity of the outer edge of the inner cylinder and υ is the kinematic viscosity of the fluid (η/ρ). The friction factor for a RAR system can be derived from the basic definition as the ratio of the drag force per unit area to the kinetic energy per unit volume, or

$$f_{RAR} = T/(0.5\pi\rho\alpha^2 R_o^2 \Omega H) \quad (3)$$

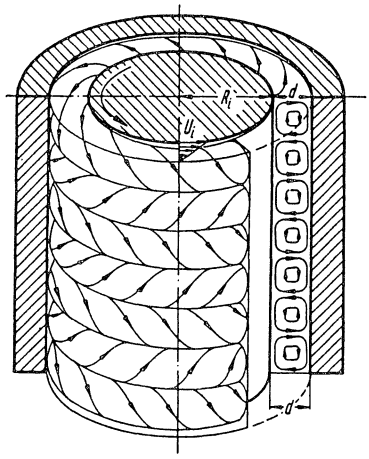


Figure 5 *Taylor vortices*

where H is the height of the cylinders. At extremely high rotational speeds, the design of the RAR with the inner cylinder rotating, can lead to Taylor vortices (Figure 5); a situation of non-ideal mixing where layers of rings of fluid vortices are formed where fluid moving to the outer cylinder wall curls and reverses direction returning towards the inner cylinder (Schlichting, 1968).

However, due to their compact size and the independence of fluid velocity on liquid flow, RAR devices are quite practical for field studies, where RARs can be found in use in the secondary oil recovery and power industries serving to monitor the onset of biofouling as indicated by an increase in fluid frictional resistance.

3.3 FLOW CELL REACTORS

Flow cell biofilm reactors have evolved due to their obvious geometrical analogy to a wide range of engineered and biomedical systems (*e.g.*, heat exchanger tubes, cardiovascular grafts, catheters, water supply lines) that experience biofilm formation under fluid flow conditions. As discussed below, for sampling and surveillance reasons, flow cells can be either circular or rectangular in cross-section. In addition, a flow cell biofilm reactor can be operated within its environmental support system either in a CSTR or a plug flow mode.

3.3.1 *Tubular Flow Cells.* A flow cell of circular cross section has obvious geometric similarities to such engineered systems as cardiovascular grafts, catheters, heat exchange tubes, and water supply lines. Circular tubes can be machined of any material of interest in any dimension to simulate field conditions. Measurement of increases in frictional resistance due to

biofilm formation can be determined easily by measuring either increases in pressure drop at constant velocity or decreases in volumetric flow rate at constant pressure drop. Incorporation of removable sampling surfaces within the flow cell wall is a design known as a Robbins device (McCoy *et al.*, 1981); Figure 6 shows a Robbins device for either circular or rectangular cross-section. An alternative sampling design, that provides a larger sampling area, consists of a number of small tube sections held tightly together by either an outer sleeve (Bryers, 1980) or a circular clamping device (Turakhia and Characklis, 1983) as seen in Figure 7.

Reynolds number for fluid flow in a circular tube is classically defined as

$$Re = vD\rho/\mu \tag{4}$$

where $Re < 2000$ are considered laminar flow and $Re > 3\text{-}4000$ are considered turbulent flow. Velocity in a circular tube, v, is defined as the ratio of the volumetric flow rate, F, to the cross-sectional area of the tube, A_x. Shear stress in a circular tube under laminar flow is defined as $\tau_{wall} = D\Delta\mathcal{P}/4L$ where $\Delta\mathcal{P}$ is the difference in the combined static pressure and gravitational forces acting on the fluid over a distance L of tube length.

3.3.2 *Parallel Plate Flow Cells.* Another form of flow cell employes a rectangular flow channel that can be either constant in cross-sectional area down the entire length of the channel or an intentional expansion in channel width can be employed to affect turbulence and controlled flow separation in the channel. Rectangular flow cells can also incorporate removable test plugs or slides in order to monitor biofilm growth; pressure ports can also be installed to monitor increases in frictional resistance. One advantage of a rectangular versus a circular flow is that on-line non-invasive microscopic observations of cell adhesion at an inner surface can be carried out without distortion using a rectangular flow cell (Figure 3). Bryers and Rudoll (1992) have also employed a rectangular flow geometry that incorporates a germanium crystal as an attenuated total reflectance (ATR) waveguide to follow with FT-IR spectroscopy the spectra of various materials accumulating at the crystal-biofilm interface.

The reader must be aware, that although both circular and rectangular flow cells can subject attaching bacterial cells to either laminar or turbulent flow conditions, with known surface velocities and shear stresses, calculation of the Reynolds Number and surface shear stresses are different for each reactor. A specific volumetric flow rate applied to a circular tube will not establish the same fluid velocity, Reynolds number, or shear stresses in a rectangular duct. Reynolds number for a rectangular duct is based on a "hydraulic diameter" defined as. . .

$$D_h = 4A_x/P \tag{5}$$

where P = wetted perimeter of the rectangular cross-section. The Reynolds number for a rectangular duct is thus defined as

$$Re_{\square} = vD_h\rho/\mu \tag{6}$$

For rectangular ducts, laminar flow exists at $Re < 2000$ and turbulent flow at $Re > 4000\text{-}5000$.

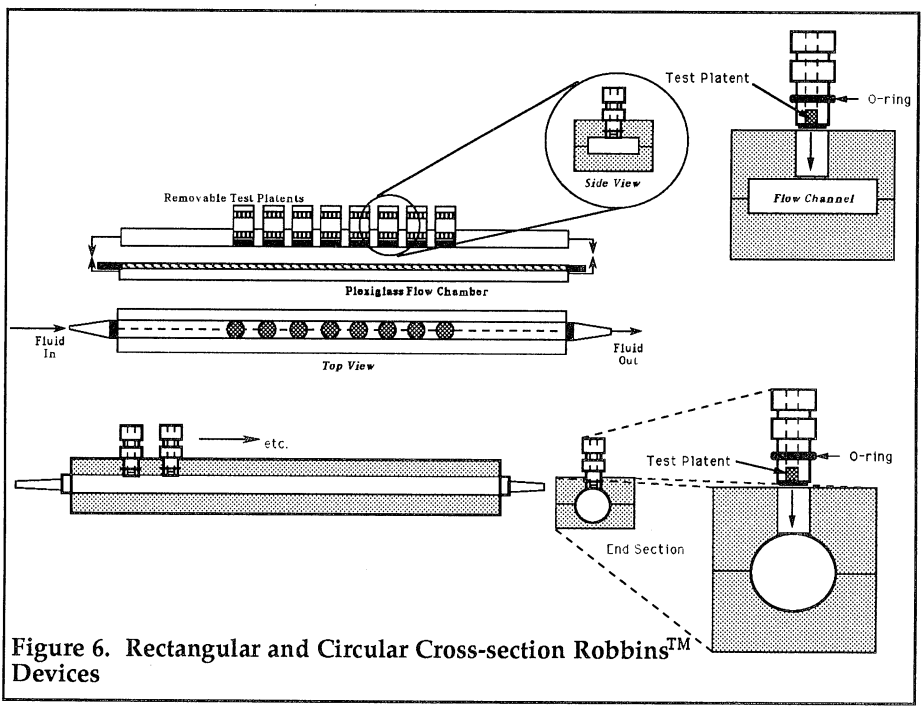


Figure 6. Rectangular and Circular Cross-section Robbins™ Devices

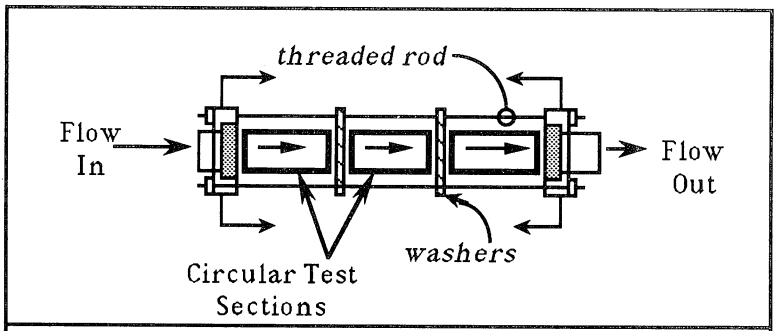


Figure 7. Biofilm flow cell of circular cross-section.

To create flow separation (*i.e.*, turbulence) in a rectangular flow cell while still at a low flow velocity, a variable cross-sectional area can be employed. To study endothelial cell responses to complex flows, one can simulate regions of flow separation observed *in vivo* using a conventional parallel plate flow chamber modified to produce an asymmetric sudden expansion (Figure 8). Briefly, the chamber consists of an upper plate of plexiglas™ with inlet and outlet ports and either a 0.02 inch or 0.01 inch recess, and an stainless steel lower plate with a recess for a glass slide containing cells. The upper plate has a recess carved into its lower face. The asymmetric sudden expansion flow path is created using a medical grade silicone rubber gasket inserted between the two plates. Thickness of the gasket sets the expansion. At the gasket edge the wall shear stress is zero and its magnitude increases with increasing distance from the gasket edge until a maximum is reached. Then, the shear stress declines, passing through zero at the separation point, until it reaches its final value downstream. The size of the recirculation zone (X), which represents the distance from the gasket edge to the point at which the wall shear stress is zero, depends upon the Reynolds number and the expansion ratio (H-h/h). In general, for a given expansion ratio, the size of the recirculation zone increases linearly with Reynolds Number until a critical Reynolds number is reached and additional

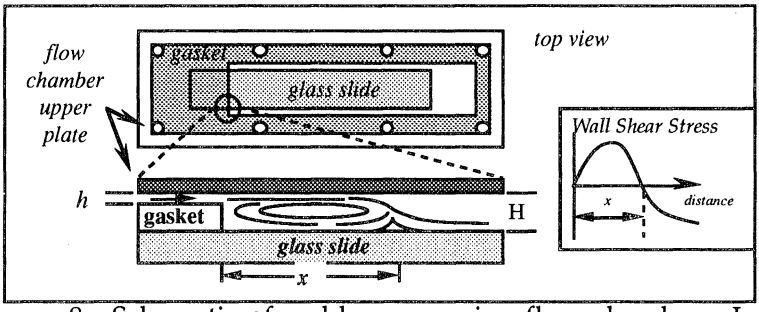


Figure 8. Schematic of sudden expansion flow chamber. Inset shows typical shear stress distribution along the glass slide.

recirculation eddies appear (Macagno and Hung, 1967; Armaly *et al.*, 1983).

3.4 RADIAL FLOW REACTOR

As first described by Fowler and McKay (1980) the reactor consists of two parallel circular disks separated by a small gap distance, about 500 μm (Figure 9). Culture media or cell suspension is delivered into the center of the bottom disk at a constant flow rate where it impinges on the upper plate then flows radially outward from the center to the outer edge where it collects in a circular manifold and leaves the system. As the total cross-sectional area for flow increases with increasing radius, the linear velocity and the fluid shear stress decrease as fluid moves away from the center. The radial flow reactor is constructed with one disk made of optically clear material so that cell adhesion and initial

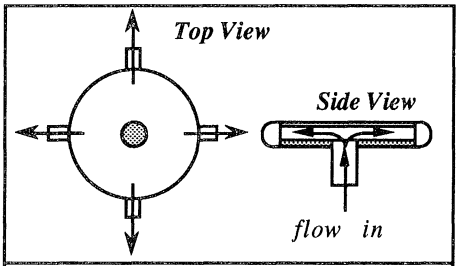


Figure 9. Radial flow reactor

biofilm formation can be observed on the bottom plate (which requires rather significant amounts of biofilm for visual observation). Since high shear forces exist nearer the center and decrease radially outward, there exists a region near the entrance where cell adhesion does not occur. This reactor design is useful in establishing that upper maximum of fluid velocity which will prevent biofilm formation. However, this reactor design is not very practical since the prerequisite hydrodynamics only prevail during initial cell adhesion and early biofilm formation. Due to the rather narrow gap width; once biofilm colonies establish, fluid flow patterns can be disturbed sufficiently to affect subsequent surface colonization. Unfortunately, the only biofilm detection method available in the original design required rather significant amounts of biofilm for visual observation; thus negating the reactor's usefulness in early cell adhesion studies. This latter shortcoming can be obviated by installing removable test surfaces randomly about the disks.

4. Biofilm System Operation and Analysis

In theory, any of the biofilm reactors above and their environmental support systems can be operated to simulate any one of the three ideal reactor concepts (*i.e.*, CSTR, Plug Flow, and Batch) presented earlier. In this section, we will present details as to how biofilm studies can be carried out in order to affect the behavior of one of these ideal reactors.

4.1 CSTR OPERATING MODE

CSTR ideality presumes complete mixing of the reaction volume, with no concentration gradients, along with a continuous input of limiting reactant and an effluent flow to affect a constant volume. Obviously, a RAR can easily accommodate these restrictions while providing sufficient surface area for the biofilm. Residence time in the RAR can be set at any value above or below the maximum growth rate of the pure or mixed culture, simply by adjusting the input nutrient volumetric flow rate. However, often in biofilm studies, it is expedient to minimize the effects of suspended cell metabolism. Thus, input nutrient flow rate can be set to establish a residence time ($\tau = V/F$) in the reactor that is much less than the maximum generation time of the culture. Consequently, any suspended biomass found in the reactor effluent can be assumed to originate by way of detachment from the biofilm. This operational technique also serves to simplify the mathematics of material balances on suspended biomass by eliminating the term for planktonic growth.

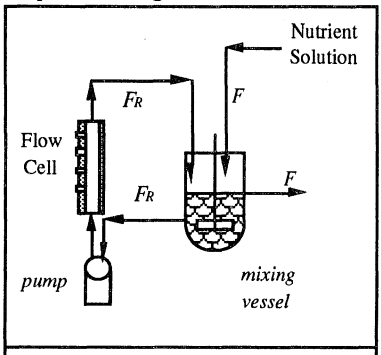


Figure 10. Biofilm flow cell operated on a recycle mode

While their geometry connotes plug flow behavior, flow cell reactors can also be operated in a CSTR mode by utilizing a fluid recycle or recirculation loop as illustrated in Figure 10. Residence time is set by the ratio of the total system volume to the inlet flow rate. By recycling a portion or all of the flow cell effluent, the axial fluid phase concentration gradients, characteristic of plug flow behavior, can be eliminated. Referring to Figure 10, to operate a flow cell reactor in the CSTR mode, one must insure that the recycle flow rate, F_R, is much greater than, F, the nutrient feed flow rate. As with all laboratory reactor systems, the degree of ideal mixing must be verified for the operating conditions in question (Levenspiel, 1972). In essence, does the real reactor behave like a CSTR, a PFR, or neither ?

4.2 PLUG FLOW OPERATING MODE

Many biofilm systems of industrial and ecological importance occur in plug flow systems, such as: biofouling of medical catheters, corrosion of pipes and sewers, or the ecology of a natural surface- or ground-water flow. Plug flow behavior in biofilm systems is characterized by longitudinal (and perhaps, radial) concentration profiles of nutrients in the liquid phase. Even if the surface of the reactor system were uniformly inoculated with cells, those attached colonies nearer the entrance of the reactor would be exposed to the highest concentration of limiting nutrient which, after time, would establish larger amounts of biofilm at the flow cell entrance. This would eventually lead to longitudinal gradients, not only in soluble nutrients, but also in attached cell concentrations. Similar spatial gradients are common to either packed bed or trickling filter reactor systems operated on a once-through basis.

Circular flow cells are obviously geometrically identical to steam condenser heat exchange tubes, water distribution lines, and wastewater sewers. The hydrodynamics of flow in a circular geometry are well defined as briefly presented above (Schlichting, 1968). Rectangular flow cells and capillaries, while providing the accessibility for microscopic observation and surface diagnostics, also have similar hydrodynamics as circular tubes (provided certain geometric criteria are met) and have been used with great success. Operation of flow cells in a plug flow mode is achieved quite simply by passing the nutrient solution (or a side stream from a operating system in the field) down the flow cell.

Plug flow behavior can also be established with a RAR, or more accurately, a number of RAR units operated in series. Theoretically, as the number of RAR units increases, the overall system performance approaches that of plug flow behavior.

5. Summary

Some of the more common reactor systems employed in the study of cell adhesion and biofilm formation under the influence of fluid flow have been described. Sampling and experimental requirements are shown to greatly influence the design and construction of a biofilm reactor. As analytical techniques evolve, the capability to non-invasively follow the development of biofilm and to assess the attached cell reactivity has increased. Both non-invasive and invasive diagnostic methods affect the type and design of biofilm flow reactor with both types of analyses providing complementary information on biofilm processes. Biofilm reactors coupled with their environmental support systems can be operated in the laboratory, *in vivo*, or in the field to simulate the ideal reactor behavior of either a CSTR, plug flow, or batch reactor; which facilitates the collection of data necessary for a complete material balance analysis of the system.

6. References

Armaly, B.F., Durst, F., Pereira, J.C.F., and Schonung, B. (1983) Experimental and theoretical investigation of backward facing step. *J. Fluid Mech.* **127:** 473-496.

Bremer, P. J. and Geesey, G. G. (1991) An evaluation of biofilm development utilizing non-destructive attenuated total refelectance FT-IR spectroscopy, *Biofouling*, **3**, 89-100.

Bryers, J. D. and Rudoll, T. (1992) unpublished data, Ph. D., Duke University.
Bryers, J. D., (1980) *Dynamics of Early Biofilm Formation in a Turbulent Flow System*, Ph.D. Dissertation, Wm. M. Rice University, Houston, TX.
Characklis, W. G. and Marshall, K. C. (Eds.) (1990) *Biofilms*, J. Wiley-Interscience, New York.
Escher, A. R. (1986) *Colonization of a Smooth Surface by Pseudomonas aeruginosa: image analysis methods*, Ph.D. Dissertation, Montana State University, Bozeman, MT.
Feurerstein, I. A. and Kush, J. (1986) Blood platelet surface interaction on fibrinogen under flow as viewed by fluorescent video microscopy, *J. Biomed., Engrg.*, **108**, 49-55.
Fowler, H.W. and McKay, A.J. (1980) Measurement of microbial adhesion in flow fluid, in R. W. Berkeley, J. M.Lench, J. Melling, Rutter, P. R., and Vincent, B. (Eds), *Microbial Adhesion to Interfaces*, Ellis Horwood, Chichester, p.163
Kornegay, B. H. and Andrews, J. F. (1967) *Characteristics and kinetics of fixed film biofilm reactors*, Final report, Grant WP-0181, Fed. W. P. C. administration, U.S. GPO, Washington, D.C.
La Motta, E. J. (1974) *Evaluation of Diffusional Resistance in Substrate Utilization by Biological Films*, Ph.D. dissertation, University of North Carolina, 1974.
Levenspiel, O. (1972) *Chemical Reaction Engineering* , 2nd Ed. J. Wiley, 143.
Macagno, E.O., and Hung, T.K. Computational and experimental study of a captive annular eddy. *J. Fluid Mech.* **28**: 43 (1967).
McCoy, W. F., W.F., Bryers, J.D., Robbins, J., and J. W. Costerton (1981) Observation of biofouling film formation, *Can. J. Microbiol.*, **27**: 910-927.
Molin, G. and I. Nilsson (1983) Effect of different environmental parameters on biofilm build-up of *Pseudomonas putida* ATCC-11172 in a chemostat, *Eur. J. Appl. Microbiol. & Biotechnol.*, **18**, 114-119.
National Science Foundation's Engineering Research Center for Microbial Interfacial Process Engineering, Montana State University, Bozeman, MT.
Picologlou, B. F. Zelver, N., and Characklis, W. G. (1980) Biofilm development and associated energy losses in water conduits, *J. Hydrol. Div., ASCE*, **106**, 733-746.
Schlichting, H. (1968) *Boundary Layer Theory*, 6th Ed., McGraw-Hill Publ., NY.
Siebel, M. A. and Characklis, W. G. (1991) Observations of a binary population biofilm, *Biotechnol. Bioengrg.*, **37**, 778-789.
Taylor, G. I. (1923) Stability of a viscous liquid contained between two rotating cylinders, *Phil. Trans. A.*, **223**: 289-223.
Turakhia, M. H. and Characklis, W. G. (1983) An observation of microbial cell accumulation in a finned tube, *Can. J. Chem., Enrgrg.*, **61**, 873-875.
Turakhia, M. H. and Characklis, W. G. (1984) Fouling of heat exchange surfaces: measurement and diagnosis, *Heat Transfer Engrg.*, **5**, 93-101.

ACKNOWLEDGEMENTS

JDB would like to thank both the National Science Foundation (BCS-9020502) and the NATO Advanced Studies Institute for their financial and intellectual support of this effort. WGC gratefully acknowledges the support of the National Science Foundation Engineering Research Centers Program and the Center's Industrial Associates.

MICROELECTRODES: A VERSATILE TOOL IN BIOFILM RESEARCH

J. C. VAN DEN HEUVEL, D. DE BEER, C. C. H. CRONENBERG
University of Amsterdam
Dept. of Chemical Engineering & Biotechnological Centre
Nieuwe Achtergracht 166
1018 WV Amsterdam
The Netherlands

1. Introduction

The use of immobilized cells is advocated for a number of bioengineering purposes, e.g. volumetric production rate, retention of expensive biomass, product quality, and convenient downstream processing. Up to now, succesfull application of such biofilms is rather empirical, since detailed knowledge of the internal structure and performance usually is not available. Due to inhomogeneous biomass density, variable kinetics, limited substrate penetration, starvation, high internal product concentrations and inhibitory effects, mathematical modelling of concentration gradients in active bioflims is of limited value. Therefore, direct measurements inside biofilms are needed to verify these models and to improve the mechanistic understanding of biofilm processes. The spatial resolution of classical analytical techniques, such as concentration measurements in extracted pore water from sliced biofilms, amounts to about 1 mm and is insufficient for biofilms with high activity and a concomitant substrate penetration depth of about 100 μm. Microelectrodes, i.e. needle-shaped devices with a sensitive tip, have proven to be most suitable and indispensable tools for these kind of measurements. Interesting studies with oxygen microelectrodes have been performed in e.g. penicillium pellets (Wittler et al., 1986) and trickling filter biofilms (Kuenen et al., 1986), revealing internal convective flows and local oxygen production by algae, respectively.

The tip diameter of a microelectrode typically should be less than 10 μm to ensure: (i) sufficient spatial resolution, (ii) negligible substrate consumption under conditions of diffusive mass transfer, and (iii) minimal mechanical disturbance of the biofilm. Because of the extremely small sensitive area, microelectrodes possess a high electrical impedance and a small capacity, which require a sensitive electrometer and shielding from electromagnetic interference. Also connected to the small dimensions, qualities like selectivity, sensitivity, and stability will be less as compared to macro-electrodes. Other characteristics like fragility and lifetime up to now have restricted the manufacturing and use of microelectrodes mainly to research environments. From the great deal of attention biosensors attract, however, considerable improvements, increasing commercial availability and routine application are anticipated in the next decade.

In this contribution the principles, manufacturing and characteristics of a potentiometric pH microelectrode and an amperometric glucose microelectrode will be presented, as well as their application and relevance in biofilm research.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 631–644.

2. Liquid-membrane ion-selective microelectrodes

2.1. GENERAL PRINCIPLES

Ion-selective liquid-membrane microelectrodes are available for a number of biotechnologically relevant compounds like H^+ (Ammann, 1981), NH_4^+ (De Beer and Van den Heuvel, 1988a), NO_3^- (De Beer and Sweerts, 1989), and HCO_3^- (Khuri et al, 1973). These electrodes are based on the well known principle that an electrical potential difference develops across an ion-selective membrane. The measuring circuit is given by:

Ag/AgCl | solution 1 | ion-selective membrane | solution 2 | AgCl/Ag

with the underlined part representing the electrode. The ideal membrane is permeable for a single ionic species, and separates two liquids containing different concentrations. Net transport through the membrane occurs in the direction of the lowest activity untill it is balanced by the electric potential difference build up across the membrane. The steady-state potential difference is given by the Nernst equation:

$$\Delta E = RT/zF \ln(a_2/a_1) = E_0 + k.\log a_2$$

where E_0 is a constant offset potential and the slope factor k amounts to about 60/z mV at 25 °C. Deviations from ideal behaviour are encountered at low activities, when additional contributions of several junction potentials and interfering ions become significant. The reference electrode junction potential can be dissipated to a negligible level by the commonly used agar bridge containing 3 M KCl.

A liquid-membrane ion-selective microelectrode consists of a glass capillary with a 1 μm tip, which is filled with a droplet of ion-exchanging liquid (LIX) as the functional membrane (Fig. 1). The LIX consists of an ion-selective ligand in a hydrophobic solvent, therefore, the tip of the capillary is made hydrophobic by silanation.

The potential across the membrane may dissipate partly through (i) the finite electrical resistance of the glass wall, (ii) an aqueous layer between the LIX and the glass wall, and (iii) the LIX itself. Obviously, miniaturization of electrodes will decrease the thickness of the glass wall, as well as the ratio of the tip surface and the inner surface of the capillary between the LIX and the glass wall. Therefore, microelectrodes more often deviate from ideal behaviour, and the slope factor k and the selectivity will be lower as compared to macroelectrodes.

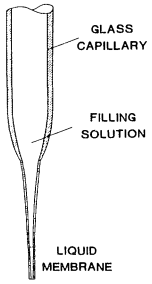


Fig. 1. *Tip liquid-membrane ion-selective microelectrode.*

Silanation is a crucial step in the preparation of liquid-membrane electrodes, since it prevents hydration of the glass wall and ensures the attachment of the LIX. The margin between insufficient silanation and plugging of the tip is small, therefore, the procedure is highly empirical and depends on glass type, silanation agent, temperature, atmospheric humidity and shape of the tip.

The membrane resistance can be optimized by a carefull choise of the membrane solvent, involving: chemical stability, inertness towards the ligand, low vapour pressure, and high solubility of ligand and membrane additives.

In actual practice, a calibration curve of the electrode is sufficient, and determination of selectivity factors is not necessary. Calibration must be done in the same solution as

used for the measurements to determine the offset potential, the slope factor k and the operational concentration range. In biotechnological experiments, solute concentrations are relatively low, i.e. activity coefficients are approximately constant, and calibration for activities is not required.

Liquid-membrane ion-selective microelectrodes can be prepared under aseptic conditions, but the membrane cannot be steam-sterilized.

Due to the small sensor surface of about 10^{-12} m^2, the electrical resistance amounts to 10^{10}-10^{11} Ω, and the input resistance of the amplifier should exceed 10^{15} Ω. The capacity of the triax connecting cable should be reduced by guarding, which improves response times considerably. Related to the small capacity of the electrode itself, the whole set-up must be shielded from electromagnetic interference by a Faraday cage and sources of electrical noise must be removed from the area.

2.2. ELECTRODE PREPARATION AND EXPERIMENTAL SET-UP

Glass capillaries (1.2 mm diameter, Clark GC120F-15) were drawn into micro-pipettes with an automatic horizontal puller (Anna puller, Biology Dept. University of Amsterdam). The outer tip diameter was about 1 μm, and their resistance when filled with 3 M KCl was 5 MΩ. Tips were immediately silanized by dipping into a 20% trimethylchlorosilane solution in CCl_4 for 15 s, followed by baking at 130 °C for 15 min. The electrode tips were filled with LIX through capillary force by dipping them into the liquid membrane solution. Subsequently, the electrode shafts were completely filled with electrolyte solution (20 mM sodium phosphate buffer pH 7.0) from a small tube. Gas bubbles were removed by heating the electrodes locally with a small heating loop. Finally, the electrode shaft was mounted in an axial connecting holder containing a 0.3 M KCl solution (liquid-liquid junction!) and a chlorinated silver wire at the end.

The ion-exchanger for the pH 4-12 range was a solution of 10%(w/v) tridodecylamine and 0.7%(w/v) sodium tetraphenylborate in o-nitrophenyl octyl ether stored in a 100% carbon dioxide atmosphere (Ammann et al, 1981). The ion-exchanger for the pH 2-7 range was a solution of 6%(w/v) 4-nonadecylpyridine and 1%(w/v) potassium tetrakis(4-chlorophenyl)borate in the same ether (Chao et al, 1988). A calibration curve for the latter electrode is presented in Fig. 2, and illustrates the almost ideal behaviour. The typical lifetime amounts to some days; sofar, we have not undertaken research to prolong this period.

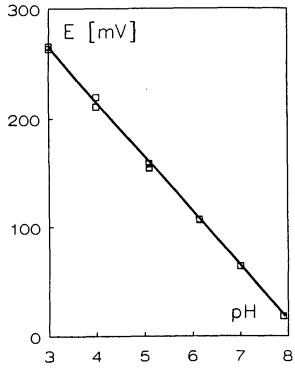


Fig. 2. *Calibration curve pH microelectrode.*

The electrochemical cell used as the measuring circuit was Ag/AgCl | 0.3 M KCl | phosphate buffer | LIX | sample | 3 M KCl agar bridge | 0.3 M KCl | AgCl/Ag. The electrode assembly was placed in a Faraday cage. Calibration and concentration measurements were done in a continuous flow cell at 30 °C. The solutions inside and outside the Faraday cage were electrically separated by tricklers. The electrical potential between the Ag/AgCl wires was conducted through a triaxial cable (Keithly 6011) to an electrometer (Keithly 617), placed outside the cage.

Spherical biofilms were fixed in the flow cell with two tiny pins. Electrodes were positioned in the biofilm by a micromanipulator driven by a stepper motor. During themeasurement of concentratation gradients in the biofilm, fresh medium was pumped through the flow cell. Prior to each experiment, the biofilms were incubated in the appropriate medium for 40 min to achieve a steady state.

2.3. APPLICATION: pH PROFILES IN METHANOGENIC AGGREGATES

2.3.1. *Introduction.* Anaerobic wastewater treatment is a multi-step process in which soluble organics are degraded to fatty acids and, subsequently, converted to methane and carbonate. Methanogenesis involves a complex interaction of various microbial species (Dubourguier et al, 1987); the digestion of acetate is responsible for 65%-96% of the methane formation (Weber et al, 1984), and is considered as the rate limiting step in the methane production from wastewater (McCarty, 1964). Therefore, knowledge of the kinetics of this process is necessary for the design and operation of methane reactors.

The Monod equation is widely used for the modelling of the anaerobic digestion process. The kinetic parameters μ_m, K_s, and Y_x are usually determined from a series of continuous flow or batch experiments with enrichment- or pure cultures (Yang and Okos; 1987). This method is very elaborate, especially with methanogens. A more serious disadvantage is the fact that in high performance bioreactors methanogens do not grow in suspension, but form aggregates with a typical diameter of 1 to 3 mm. Therefore, suspended growth experiments can be considered as studies on artifacts. Conversely, the aim of this study was to determine the kinetic parameters of the methanogenic acetate conversion on intact aggregates.

In active bacterial aggregates, profiles of substrate and product will develop. In methanogenic aggregates also pH profiles are formed by the conversion of acetic acid into the weaker carbonic acid. These profiles depend on the size of the aggregate and the complex interaction of diffusion, kinetics, and the distribution of the bacterial activity. Since the kinetics and the pH profile are interdependent, calculations on this system are very complex. In this study the pH profiles in methanogenic aggregates were actually measured, and this information was used to calculate substrate profiles and conversion rates.

Comparison of measured conversion rates with calculations using a structured mathematical model enabled the estimation of the relevant growth parameters, and the significance of pH profiles in aggregates for reactor operation could be evaluated.

2.3.2. *Materials and Methods.* Methanogenic aggregates were sampled from a pilot-scale Internal Circulation reactor (Paques BV, Balk, The Netherlands) treating brewery wastewater (Heineken BV, 's-Hertogenbosch, The Netherlands). Measurements on intact aggregates were performed in a mineral medium (De Beer et al, 1992) containing 0.5 mM NaH_2PO_4, supplied with the desired concentration of acetate, flushed with N_2, and adjusted to pH 7.0. Activity tests were done in fed-batch mode, micro-profiles were obtained during continuous-flow operation. The mean diameter of a single aggregate was determined with a vernier callipers, the Sauter mean diameter of a sample was obtained from an image analyzing system (Shieh et al, 1981). The effectiveness factor η was determined from the increase of the conversion rate upon disintegration of aggregates in separate cells or fragments smaller than 50 μm, as achieved in two hours with a magnetic stirrer at 250 r.p.m. All other analytical (De Beer et al, 1992) and experimental methods (De Beer and Van den Heuvel, 1988b) have been reported previously.

Substrate profiles in the spherical aggregates were calculated as described earlier (Van den Heuvel, 1992). As the acetate concentration range applied is well below the substrate inhibiting concentration (Speece, 1988), simple Monod kinetics were used and unionized acetic acid was considered the actual substrate (Andrews and Graef, 1973.). This was effected by taking $K_s = K_{s,pH=7} \cdot K_a/H^+$ referring to the total concentration of both forms of acetate. The experimental relation between μ_m and the pH (Van den Berg et al, 1975) was used, and approximated by a Gaussian curve: $\mu_m = \mu_{m,pH=7} \cdot \exp[-0.5((pH - 7)/0.62)^2]$. The effective diffusion coefficient D_e of acetate inside the aggregates was conservatively assumed to be 4.2×10^{-10} m^2/s (Speece, 1988), which is 34% of the value in pure water (Perry and Green, 1984), Y_x was set at 1.5 g biomass dry weight/mol acetate (De Zeeuw, 1984).

2.3.3. *Results.* Methane production rates of aggregates in fed-batch are presented in Fig.3, showing maximal conversion rates were reached at 15 - 20 mM acetate. In an additional experiment with 10 mM phosphate buffer at pH=7, no significant influence of the acetate con-centration was observed, and the maximal conversion rate was already reached at 5 mM (data not shown).

Since the growth parameters μ_m and K_s strongly depend on the local pH value, pH profiles in 50 aggregates were measured at various acetate concentrations in the bulk liquid under steady-state conditions. The average characteristic diameter of these aggregates was 1.64 mm, and close to the Sauter mean of the total sample of 1.58 mm. In Fig. 4 a few characteristic pH profiles are presented. As could be expected, the pH inside aggregates was higher than the bulk pH. In the absence of acetate in the bulk liquid pH gradients, although very small, were also observed. In the concentration range upto 10 mM, relevant for most anaerobic reactors, the maximal pH value inside the aggregates increased with higher acetate concentrations in the bulk. Further increase of the acetate concentration only caused a slight increase of the internal pH. At an acetate concentration of 5 mM the pH increased to a depth of 280(±40) μm, while a plateau was apparent in the centre. At acetate concentrations of 10 mM or higher, a pH increase was observed down to a larger depth of 410(±60) μm. In aggregates incubated in medium buffered with 10 mM phosphate, no pH gradients developed (data not shown).

The measured pH profiles were used to calculate acetate concentration profiles and conversion rates in aggregates. For this purpose simplified mean pH profiles were composed as given in Fig. 5, including the observed diffusive liquid boundary layer of 50 μm thickness (see Fig. 4). By comparing calculated conversions at various acetate concentrations in the bulk liquid with the fed-batch measurements, a fit based on methane production was found with $\mu_{m,pH=7} = 2.1 \times 10^{-7}$ s^{-1} and $K_{s,pH=7} = 2.6$ mM (Fig. 3, curve C).

Calculations of the local activities in the aggregates are pictured in Fig. 6 (solid lines), showing that even for homogeneously distributed active biomass, the conversion rate strongly depended on the location, although acetate penetrated the whole aggregate. It was calculated that the acetate concentrations in the centre of aggregates incubated in 5, 10 and 20 mM acetate amounted to 3.73, 8.59 and 18.33 mM, respectively. The the local conversion rates calculated without pH profiles (dashed lines), show that the pH value is of much greater importance than the acetate concentration. Although the activity of the total aggregate is positively related to the substrate concentration, Fig. 6 shows that the activity in the centre of the aggregates even may decrease at higher substrate concentrations due to the more pronounced pH profile.

In Fig. 3 measured and calculated conversion rates of a single aggregate are presented, to

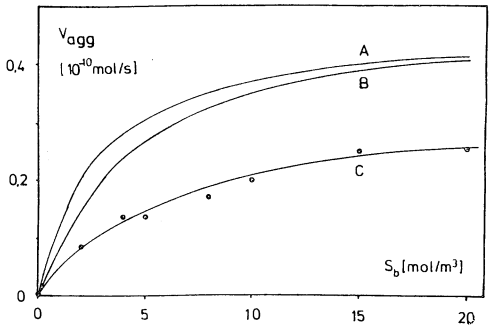


Fig. 3. *Conversion rate of single aggregates in fed-batch culture (●). Calculated with diffusion resistance for (A) none, (B) acetate only, (C) acetate and protons; pH profiles from Fig. 5.*

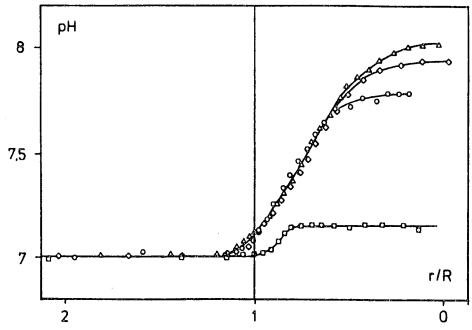


Fig. 4. *pH microprofiles measured in methanogenic aggregates with a characteristic diameter of 2.2 mm, incubated in acetate 0 mM (□), 5 mM (○), 10 mM (◇) and 15 mM (△).*

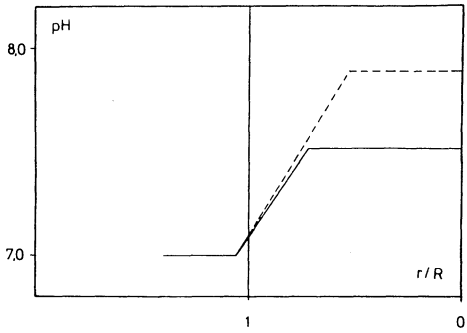


Fig. 5. *Simplified pH profiles used for calculations on transport and conversion in methanogenic aggregates, at acetate concentrations of 5 mM (solid line) and 10 mM or more (dashed line).*

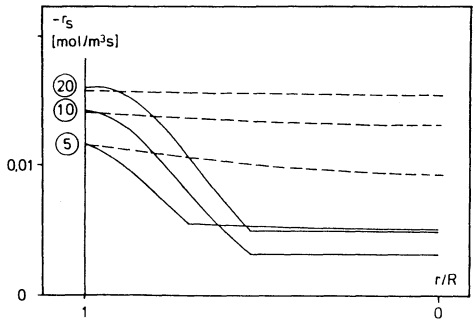


Fig. 6. *Calculated activity profiles in 1.64 mm methanogenic aggregates, with (solid line) and without (dashed line) pH gradients. Encircled numbers indicate the acetate bulk concentration.*

establish the influence of the mass transfer resistance for acetate and the role of pH profiles on the conversion rate and the effectiveness factor. From comparison of curve (A) and (B) it is clear that the influence of acetate transport limitation by external and internal diffusion resistances is insignificant for the conversion rate in aggregates. Comparison of curve (B) and (C) shows that the local pH values determine the conversion rate to a much larger extend.

Table 1 presents effectiveness factors η calculated with the structured model, both inclusive as well as exclusive the effect of pH profiles, together with values determined from the increase of the conversion rate upon disintegration of aggregates.

2.3.4. *Discussion.* The effectiveness factors η in Table 1 are almost independent of the substrate concentration in the bulk, while the measured values (column C) were far from unity. The explanation is that the influence of acetate transport resistance on the conversion rate is negligible for the relevant S_b range. Even at high substrate concentrations η did not approach a value of 1, since the elevated internal pH value reduced μ_m and thus the maximal conversion rate. The extent of the pH profiles depends on the size of the aggregate. The value of η of disintegrated aggregates does amount to 1 because even in the largest fragments (50 μm) no significant pH profiles can develop. The effectiveness factors obtained from disintegration experiments are much lower than those calculated with fixed kinetic parameters (no pH profiles taken into account), but are in good agreement with the calculations using pH dependent kinetics and comprising the pH profiles. Therefore, it can be concluded that the model is reliable and pH profiles are of great importance for the behaviour of methanogenic aggregates.

Reactor behaviour is expected to be strongly influenced by the pH profiles occurring in aggregates. A decrease of the bulk pH may result in a lower internal pH and a concomitant decrease of the local K_s value, leading to a higher biomass activity, especially at low substrate concentrations. Increase of the influent pH buffer capacity may have the same effect. Moreover, this enables establishment of an optimal pH value for μ_m in the whole aggregate.

S_b	effectiveness factor η		
(mM)	A	B	C
5	0.92	0.57	0.62
10	0.96	0.62	0.42
15	0.98	0.63	0.61
20	0.99	0.63	0.53

Table 1. *Effectiveness factors η of aggregates at various acetate bulk concentrations (A) calculated using the structured model when no pH profiles occur, (B) calculated using the structured model including pH profiles and (C) values obtained from the increase of activity upon disintegration into cells.*

The measurements of the pH profiles revealed a liquid boundary layer of about 50 μm, increasing the surface pH value with approximately 0.1 unit. Thereby it influenced the pH profiles inside the aggregate to some extent and thus the kinetics. An acetate boundary layer also might influence the transport rate of substrate towards the biofilm. However, since the influence of the acetate transport rate on the conversion rate was negligible, the boundary layer for acetate was not significant. Since the pH boundary layer was small it is unlikely that enhancement of external transport by mixing will increase the conversion rate significantly in methanogenic reactors.

At the moment reliable acetate selective microelectrodes are not available. However, such an electrode would not enable measurement of unionized acetic acid, the actual substrate for methanogenesis, which is determined by the total acetate concentration and the pH. Therefore, information on the acetate profiles without knowledge of the local pH is not sufficient. Moreover, according to calculations with the structured model, the acetate concentration in aggregates remains almost constant and is close to the bulk concentration.

The medium pH buffer capacity strongly determined the macrokinetics of methanogenic aggregates by its influence on the pH profiles in aggregates. A phosphate concentration of 10 mM completely prevented the development of pH profiles in aggregates, and no influence of the substrate concentration on the conversion was found in the substrate concentration range from 5 to 30 mM, due to the low local K_s values. As a consequence, the theoretical prediction of Henze and Harremoës (1983) that mass transfer resistance in methanogenic biofilms is not significant, is only valid in well buffered media. The verification of this prediction by Dolfing (1985) was based on experiments in media buffered with 5 mM phosphate, in which the pH profiles will be strongly reduced.

The conversion rate in aggregates is influenced by the transport resistance for protons. This can be understood by the notion that under physiological conditions proton concentrations are very low, in aggregates varying from 0.01 to 0.1 μM, and 6 orders of magnitude smaller than the acetate concentration. The mass transfer resistances are sufficiently large to maintain small proton concentration gradients (maximal value 2×10^{-1} mol/m^4), nevertheless leading to significant pH gradients. Limitation of the conversion rate by acetate depletion in the aggregate under typical reactor conditions, however, requires concentration gradients of at least 2×10^{3} mol/m^4, for which the mass transfer resistance is obviously insufficient. From this study it can be concluded that mass transfer resistance is important in methanogenic aggregates via the influence of the internal pH on the kinetics. This conclusion is relevant for most industrial reactors, since the phosphate concentration is usually well below 1 mM. The bulk liquid of the Internal Circulation reactor, from which the investigated aggregates were obtained, is weakly buffered at a pH value of 6.8 to 7.1 by approximately 0.1 mM phosphate and 20 - 25 mM carbonate. It was calculated that at the typical acetate concentration in the reactor of 5 mM, the acetate concentration in the centre of aggregates amounts to 3.73 mM. Using an alkalinity balance, it can be calculated that in this medium the pH in the centre of aggregates amounts to 7.4, which is in good agreement with the presented microelectrode measurements.

The high internal pH reduced the activity in the centre of the aggregates to 15 - 30% of the activity in the peripheral zones. The volume of the central part of aggregates ($r/R<0.5$) is only 10% of the total volume, therefore, an additional assumption of a non-homogeneous biomass distribution, i.e. an inactive centre, is insignificant for modelling.

Previous measurements on aggregates from an upflow anaerobic sludge blanket reactor, fed with incompletely acidified wastewater, showed the presence of an acidifying process (De Beer and Van den Heuvel, 1988b), resulting in pH profiles strongly deviating from those reported in this study. Moreover, pH profiles depend on the size of the aggregates, larger aggregates having more pronounced pH profiles. Since both the processes inside aggregates and their size distribution may depend on the reactor type and influent composition, the reported pH profiles may not be valid for aggregates from other reactors.

2.3.5. *Conclusion.* Knowledge of K_s and μ_m only, is not sufficient to understand the behaviour of a methane reactor containing aggregates. This study shows that pH profiles play a central role and a structured model is required, comprising these pH micro-profiles and the pH dependency of the kinetics. Since in most microbial conversions net proton consumption or -production takes place, this conclusion seems relevant for many processes in which biofilms are involved.

3. Amperometric enzyme microelectrodes

3.1 GENERAL PRINCIPLES

The specific catalytic properties of enzymes make them highly suitable as selective and sensitive element in analytical devices for the detection of their substrates. Since Clark described the first enzyme-based sensor for glucose in 1962, considerable progress has been made for a wide variety of substrates (e.g. urea, ethanol, lactate, sucrose) and applications (Scheller et al, 1985). In electrodes the catalysed reaction rate is made proportional to the substrate concentration, and either the rate of product formation or reactant consumption is measured. When these components are electroactive, their concentration can be monitored directly. Enzymes applied typically are oxidoreductases, and common systems are based on the detection of oxygen consumption (being the co-substrate) or the production of hydrogen peroxide.

Electrodes of Pt, Au or C are often used as sensing element or 'transducer' to convert the chemical signal into an electric current. The electrode surface is provided with a 'selector' by a coating of immobilized enzyme. Immobilization of 1 μm enzyme layers on microelectrodes can be achieved by direct adsorption on the surface, or covalent cross-linking with glutaraldehyde (Yao, 1983). Other immobilization techniques result in much thicker membranes. Covalent binding of the enzyme layer onto the electrode surface can be obtained after silanation. Chemical and mechanical stability of the enzyme membrane may be improved substantially by addition of another protein like bovine serum albumin (BSA). The purpose of these immobilisation techniques is to make a thin, highly active and stable enzyme membrane to obtain a fast and sensitive electrode with a long lifetime.

Although most enzymes follow non-linear Michaelis-Menten kinetics, a linear electrode response can be obtained over several orders of magnitude, by imposing a sufficiently large diffusion barrier. To this end, the diffusion coefficient of the substrate in the enzyme membrane itself, or in an additional external membrane, should be 10 - 100 times smaller than in the sample medium. As a consequence, such electrodes exhibit no stirring effect, and can be used in biofilms under conditions of diffusive mass transfer.

In glucoseoxidase-based electrodes with a positive potential of 0.7 V with respect to a Ag/AgCl reference electrode, the following reactions take place (see Fig. 7):

$$\text{glucose} + O_2 \rightarrow \text{gluconate} + H_2O_2$$

$$H_2O_2 \rightarrow 2\,H^+ + O_2 + 2\,e^-$$

Glucose and oxygen diffuse from the sample medium into the periphery of the enzyme membrane and are converted into gluconate and hydrogen peroxide. These products diffuse both in the direction of the medium and the electrode surface, the latter flux being smaller. Hydrogen peroxide is immediately oxidised at the Pt electrode, resulting in a surface concentration equal to zero, and the electric current is proportional to its flux and production rate. The equimolar oxygen flux generated, only partly replinishes the oxygen consumed by the enzyme. Depending on the aeration, pH and buffer capacity of the medium, the developing oxygen and pH profiles

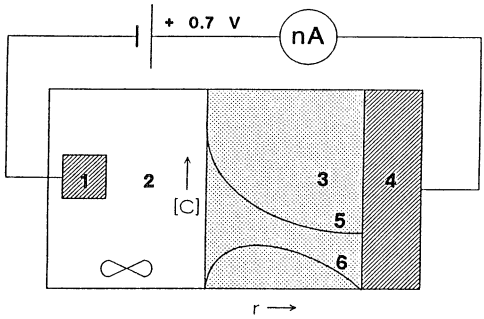


Fig. 7. *Schematic diagram of the glucose microelectrode: (1) Reference electrode, (2) Bulk liquid, (3) BSA cross-linked glucose-oxidase membrane, (4) Positive Pt electrode, (5) Glucose concentration profile in the membrane, (6) H_2O_2 concentration profile in the membrane.*

may have a great influence on the electrode response. Obviously, the measuring conditions should be know and a proper calibration procedure is required.

Application of an additional (protective) outer membrane as a diffusive resistance will decrease the glucose flux, and relatively increases the peroxide flux to the electrode surface. This may result in a comparable, or even larger, response at a longer reponse time. The higher peroxide concentration in the enzyme membrane, however, leads to a faster deactivation of the enzyme. Recently, Koopal (1992) has succeeded in the construction of a microsensor based on the direct interaction of glucose oxidase and a conducting polymer polypyrrole (i.e. hydrogen peroxide is no longer produced), and claims an operating lifetime of more than two month.

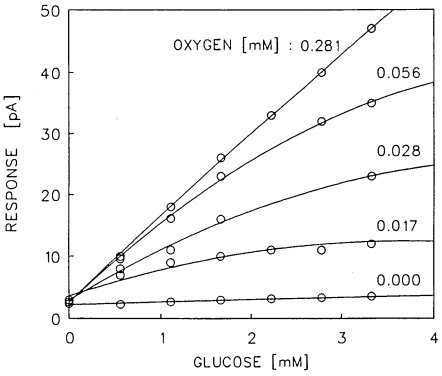
The selectivity of enzyme electrodes mainly depends on the enzyme itself. Most enzymes, except alcohol and amino acid oxidases, are very specific. For example, glucose oxidase is 5.10^4 times less active with other sugars, such as fructose, lactose or sucrose. Interference is obtained largely by electroactive species which can diffuse to the sensor surface to be oxidised at the relatively high potential required for the oxidation of hydrogen peroxide.

The general remarks made earlier on microsensors to be used in biofilms, apply equally well to the miniaturization of enzyme electrodes. Thereby, the immobilization technique to produce enzyme membranes of about 1 μm thickness is of crucial importance. The sensitivity is expressed as current per tip area at unit substrate concentration. Typical values amount to 0.1 - 1 A/m^2 at 1 mM glucose, i.e. measuring currents lie in the pA-range. A small offset current is found at zero concentration; response times are in the order of seconds. The drift of the signal typically amounts to 2% h^{-1}, and is positively correlated to the substrate concentration. Although more complicated in design and construction than ionselective microelectrodes, amperometric enzyme micro-electrodes are less sensitive to electromagnetic interference. The detection level is determinded by the signal noise, and can be as low as 5 μM glucose.

3.2 PREPARATION OF AN OXYGEN INDEPENDENT GLUCOSE MICROELECTRODE

In biofilms the local oxygen concentration may vary strongly, therefore, the application of conventional microelectrodes based on the enzymic oxidation of glucose by glucose oxidase is limited (Cronenberg and Van den Heuvel, 1991). In the electrode described here, the sensing surface is not in direct contact with the sample, but located in a micro-environment oxygenated internally (see also Fig. 9a).

A 0.1 mm platinum wire (Drijfhout, Amsterdam, NL) was etched electrically to obtain a conical shape with a tip of about 1 - 2 μm. The wire was inserted into a glass capillary (AR glas, Schott) with a diameter of 1 mm. Subsequently, the glass was melted, pulled and fused to the wire. The tip was polished with Diaplast particles (0.25 μm, Winter, Hamburg, FRD) to obtain a flat surface. The platinum core was etched back a few μm and platinized in a 3% w/v hexachloroplatinic acid (Aldrich) solution at +450 mV versus a saturated calomel electrode for



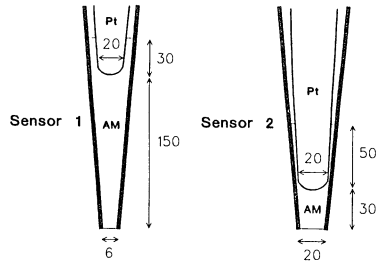


Fig. 8. *Calibration 10 μm ECPM at different oxygen concentrations.* **Fig. 9a.** *Schematic design sensors 1 and 2, AM=agar membrane; distances in μm.*

40 to 90 s, upon which a porous platinum matrix was formed at the electrode tip. The electrode was dried, placed in a 99% 3-aminopropyltriethoxy silane solution for two hours, and washed with toluene. Subsquently, the ultimate tip was placed in a freshly prepared enzyme mixture with 10% w/v glucose oxidase (from *Aspargillus niger*, grade I, Boehringer Mannheim) and 3% glutaraldehyde. After 15 minutes incubation, the electrode was dried for one hour. After repeating the incubation and drying procedure 2 - 4 times, the enzyme coated platinum microelectrode (ECPM) was ready. A calibration graph at different oxygen concentrations is given in Fig. 8.

A glass tube with a diameter of 4 mm was melted and pulled to a pencil shaped capillary with a tipdiameter of 5 - 25 μm. The tip of the capillary was dipped into a 1.2 % w/v agar (extra pure, Merck) solution of 50 °C, resulting in the formation of an agar membrane inside the tip of 50 - 150 μm thickness, depending on the time of immersion. The ECPM was inserted into the glass capillary and the agar membrane. After positioning the ECPM at the desired distance from the tip opening, the shaft was fixed with glue and the fully assembled microsensor was ready for use.

Two electrode configurations designed for different analytical ranges are presented in Fig. 9a, while their calibration curves are given in Fig. 9b. The lifetime of these microelectrodes amounts to some month; obviously, they cannot be steam-sterilized. Measurements were carried out in a 0.1 M phosphate buffer of pH 6.8, the oxygen concentration in the media was adjusted by bubbling through air, oxygen or pure nitrogen, and monitored with a commercial oxygen probe (YSI, model 5331). After the measurements, the sensor is to be stored in water saturated air at room temperature to prevent the agar from drying. Other experimental details are published elsewhere (Cronenberg et al, 1991).

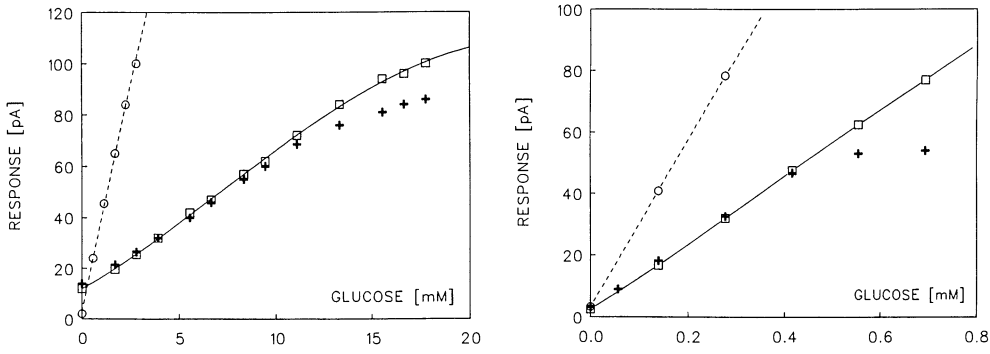


Fig. 9b. *Calibration graphs (solid lines) for sensor 1 (left) and sensor 2 (right), (□) air-saturated conditions, i.e. 0.28 mM O_2, (+) under N_2-atmosphere, (o) original EPCM under air-saturated conditions.*

3.3. APPLICATION: GLUCOSE DIFFUSION COEFFICIENTS IN BIOFILMS

3.3.1. *Introduction.* Effective diffusion coefficients (D_e), including porosity and tortuosity, are important parameters for the mathematical modelling of bioflims. Diffusion of rate-limiting substrates is of special concern, therefore, values for D_e of glucose in several biofilms have been reported (Karel et al, 1985). These were obtained mainly by imposing a known glucose concentration on the boundary of the biofilm, and measurement of either the steady-state (Matson & Charaklis, 1976), or the transient glucose flux into the biofilm (Hannoun and Stephanopoulos, 1985; Tanaka et al, 1984). Both methods are indirect, as they are based on concentration changes in the bulk liquid. This contribution is aimed at the determination of D_e of glucose by direct measurements inside biofilms, using an oxygen- and pH-independent micro-electrode (Cronenberg et al, 1991). A well-known model system of yeast cells immobilized in spherical gels was used under both transient- and steady-state conditions.

With the transient state method, concentration changes at a fixed position inside a deactivated biofilm were measured upon a stepwise concentration change in the bulk solution. According to the steady-state method, the ratio of the glucose gradient on both sides of the liquid-solid interface of the glucose consuming biofilm was determined. The latter value is highly significant for D_e of the outer, i.e. most important layer of the biofilm.

3.3.2. *Materials and Methods.* The biocatalyst model system was prepared from a mixture containing 2% w/v agar (extra pure, Merck, Darmstadt, FRG) and 2% w/v or 15% w/v dried baker yeast (*Saccharomyces cerevisiae*, type II, Sigma, St Louis, USA), corresponding to a volume fraction of 0.04 and 0.3, respectively. At 40 °C, the mixture was trickled into highly liquid paraffine oil at room temperature to form spherical gels with a diameter of 3.3-3.5 mm.

All measurements were performed at 30 °C. The activity of the immobilized yeast cells was determined on a batch of gel beads, incubated in a mineral medium containing 5.5 % minerals (Evans et al, 1970) and the desired glucose concentration, adjusted to pH 7.0. The macroscopic conversion rate of the gelbeads was measured with a glucose macrosensor (Yao, 1983).

Microelectrode measurements were performed on single gel beads fixed in a well-mixed aerated measuring vessel. For the transient-state measurements, the yeast cells were first deactivated by incubation in 0.1% w/v $HgCl_2$ for 2 h. After flushing the gels in demineralized water, the micro-electrode was positioned in the centre of the bead. After an incubation period of 45 min in aerated 0.1 M phosphate buffer, glucose was added and the electrode response in time was recorded. Effective diffusion coefficients of glucose were obtained by fitting the time dependent concentration near the centre of the initially glucose-free bead, according to Crank (1973):

$$\frac{S}{S_b} = 1 + 2\frac{R}{\pi r}\sum_{n=1}^{\infty}\frac{(-1)^n}{n}\sin\left(\frac{n\pi r}{R}\right) \times \exp\left(-\frac{D_e n^2 \pi^2 t}{R^2}\right)$$

For the steady-state measurements, freshly prepared gel beads containing 2% yeast were first incubated in a medium containing 2.8 mM glucose for 1 h, during which no growth occurred. Contrarily, the beads containing 15% yeast were incubated in demineralized water, to prevent those beads of becoming too active for accurate gradient measurements. Subsequently, beads were incubated in the measuring vessel in the desired medium for 40 minutes. Thereupon, a glucose profile was measured with the micro-electrode. After each measurement the calibration of the electrode was checked for drift. D_e was obtained by comparison of the glucose concentration gradient at both sides of the liquid/gel bead interface. From the unequivocal flux through the interface, the following relation holds:

$$J = -D_e(dC/dr)_{internal} = -D(dC/dr)_{external}$$

3.3.3. *Results and discussion.* For the transient measurements the stirring rate was chosen sufficiently high, so the concentration at the surface of the bead could be considered to equal the bulk liquid concentration. Reproducible transients were obtained with step-up experiments from 0 to 1.11 and 2.22 mM glucose, respectively, and are given in Fig. 10. With beads containing 2% deactivated yeast, a good fit was obtained for $D_e = 6.7 \times 10^{-10}$ m^2/s, which is close to the value in water, viz. 6.8×10^{-10} m^2/s (Tanaka et al., 1984). Since the position of the sigmoid curve strongly depends on small differences in D_e, the sensitivity of the of the method is high. Furthermore, the reliability of the experimental result is improved by the additional two-point calibration of the enzyme electrode inside the biofilm at the initial and final substrate concentration. Notably, a value for D_e in the model system could be obtained within 1 hour.

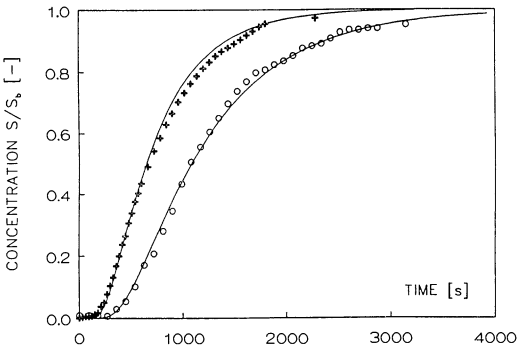


Fig. 10. *Glucose transients in gel beads: (+) 2% yeast, (o) 15% yeast. Solid lines: theoretical curves.*

Application of this elegant method for other types of biofilms, though, is limited to those with a well defined shape which can be described mathematically.

Beads containing 15% deactivated yeast had a much lower effective diffusion coefficient of 3.7×10^{-10} m^2/s, being within the range specified by Furusaki and Seki (1985) for yeast immobilized in alginate beads. This illustrates the usefulness of the glucose micro-electrode to verify models describing diffusive transport in biofilms. Some properties of the transient method are summarized in Table 1, and compared to the steady-state method.

Table 2. Comparison of the transient- and steady-state method for the determination of effective diffusion coefficients in biofilms with a glucose micro-electrode.

Transient-state method	*Steady-state method*
1. The D_e obtained reflects the rate of glucose redistribution in the biofilm.	1. The D_e thus obtained reflects an effective diffusive permeability (Libicki et al, 1988).
2. Since the whole biofilm particle contributes to the transient concentration in the centre, D_e reflects an overall diffusion coefficient.	2. D_e indicates a more or less local diffusion rate in the outermost, i.e. most important layer of the biofilm.
3. Cells have to be inactivated, which may cause artifacts.	3. D_e is determined under actual reaction conditions.
4. Can be used only for biofilms with a well defined shape, e.g. spherical.	4. Can be used also for irregularly shaped biofilms.
5. The sigmoid shape of the theoretical curves enables high precision fits of the measured transients, i.e. the sensitivity can be high.	5. Since only a difference in the slope of a gradient exceeding 10 % can be observed, the sensitivity of this method is low.
6. A simple enzyme coated Pt microelectrode is sufficient.	6. An oxygen- and pH-independent glucose microelectrode is required.

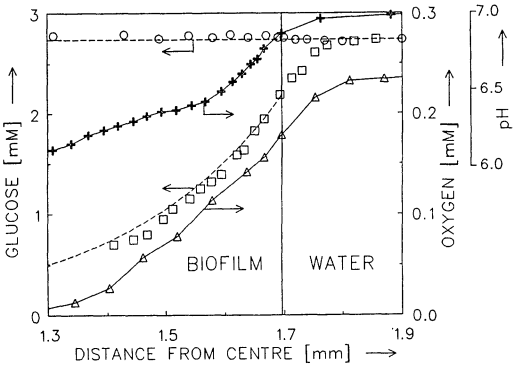


Fig. 11. *Microprofiles in 2% yeast bead, (□) glucose, (o) glucose after deactivation, (---) predicted glucose profiles, (△) oxygen, (+) pH.*

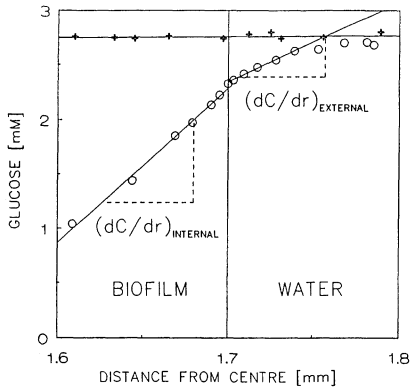


Fig. 12. *Microprofiles in a 15% yeast: (o) low activity bead, (+) inactivated bead.*

The steady-state micro-electrode measurements of glucose in a bead containing 2% yeast are given in Fig. 11; concomitant pH and oxygen profiles are also given. As no significant change in the slope was apparent at the interface, the effective diffusion coefficient was considered to be the same as the diffusion coefficient of glucose in water. The flux obtained from the slope of the gradient at the interface amounted to 4.8×10^{-6} mol/m^2.s. From the total conversion obtained with a batch of gel beads, a glucose flux of 5.1×10^{-6} mol/m^2.s could be calculated at the surface of the perfectly shaped beads. Since the micro-electrode result is in good accordance with the macroscopic result, it can be concluded that this method can be used also for the determination of glucose fluxes into irregularly shaped biofilms.

For the beads containing 15% yeast, a significant change of the interfacial slope was observed, as can be seen from Fig. 12. From these slopes a value for D_e/D of 0.5 was calculated. Consequently, D_e amounted to 3.4×10^{-10} m^2/s, which is close to the result obtained with the transient-state method.

3.3.4. *Conclusion.* The glucose micro-electrode displayed a consistent behaviour in a biofilm of immobilized yeast in agar beads. With both a steady- and a transient-state method, it was possible to determine effective diffusion coefficients of glucose directly inside the biofilm. Therefore, it is concluded that the micro-electrode described is a powerful tool for mechanistic research in biofilms or other systems where glucose distributions or fluxes on a microscale are required.

4. References

Ammann D, Lanter F, Steiner R.A, Schulthess P, Shio Y, Simon W. (1981) *Neutral carrier based hydrogen-ion selective microelectrode for extra- and intracellular studies.* Anal. Chem, 53, 2267-2269

Chao P, Ammann D, Oesch U, Simon W, Lang F. (1988) *Extra- and intracellular hydrogen ion-selective microelectrode based on neutral carriers with extended pH response range in acid media.* Pfluegers Arch, 411, 216-219

Crank J. (1973) *The mathematics of diffusion.* Oxford University Press, London.

Cronenberg C.C.H, Van Groen H, De Beer D, Van den Heuvel J.C. (1991) *Oxygen-independent glucose microsensor based on glucose oxidase.* Anal. Chim. Acta, 242, 275-278

Cronenberg C.C.H, Van den Heuvel J.C. (1991) *Determination of glucose diffusion coefficients in biofilms with micro-electrodes.* Biosens. Bioelectron, 6, 255-262

De Beer D, Sweerts J.P.R.A. (1989) *Measurement of nitrate gradients with an ion-selective microelectrode.* Anal. Chim. Acta, 219, 351-356

De Beer D, Van den Heuvel J.C. (1988a) *Response of ammonium-selective microelectrodes based on the neutral carrier nonactin.* Talanta, 35, 728-730

De Beer D, Van den Heuvel J.C. (1988b) *Gradients in immobilized biological systems.* Anal. Chim. Acta, 213, 259-265

De Beer D, Huisman J.W, Van den Heuvel J.C, Ottengraf S.P.P. (1992) *The effect of pH profiles in methanogenic aggregates on the kinetics of actetate conversion.* Water Res, 26, in press

De Zeeuw W. (1984) *Acclimatization of anaerobic sludge for UASB-reactor start-up.* PhD Thesis, Agricultural University Wageningen, The Netherlands

Dolfing J. (1985) *Kinetics of methane formation by granular sludge at low substrate concentrations - The influence of mass transfer limitation.* Appl. Microbiol. Biotechnol, 22, 77-81

Dubourguier H.C, Prensier G, Albanac G. (1987) *Structure and microbial activities of granular anaerobic sludge.* In: Granular anaerobic sludge; microbiology and technology (G. Lettinga, A.J.B. Zehnder, J.T.C. Grotenhuis, L.W. Hulshoff Pol, eds.) Pudoc, Wageningen, The Netherlands, 18-33

Evans C.G.T, Herbert D, Tempest D.W. (1970) *The continuous cultivation of micro-organisms: construction of a chemostat.* In: Methods in Microbiology, Vol.2 (Norris J.R, Ribbons D.W, eds.) Academic Press, London, 310-313

Furusaki S, Seki M. (1985) *Effect of intraparticle mass transfer resistance on reactivity of immobilized yeast cells.* J. Chem. Eng. Japan, 18, 389-393

Graef S.P, Andrews J.F. (1973) *Mathematical modelling and control of anaerobic digestion.* AIChE Symposium series, 136(70), 101-131

Hannoun B.J, Stephanopoulos G. (1985) *Diffusion coefficients of glucose and ethanol in cell free and cell-occupied calcium alginate membranes.* Biotech. Bioeng, 28, 829-835

Henze M, Harremoës P. (1983) *Anaerobic treatment of waste-water in fixed film reactors - a literature review.* Wat. Sci. Technol, 15, 1-101

Karel S.F, Libicki S.B, Robertson C.R. (1985) *The immobilization of whole cells: Engineering principles.* Chem. Eng. Sci, 40, 1321-1354

Khuri R.N, Bogharian K.K, Agulian S.K. (1973) *Intracellular bicarbonate in single skeletal muscle fibers.* Pfuegers Arch, 349, 285-294

Koopal C.G.J. (1992) TNO Nutr. & Food Research, Zeist, The Netherlands, personal communication

Kuenen J.G, Jorgensen B.B, Revsbech N.P. (1986) *Oxygen microprofiles of trickling filter biofilms.* Water Res, 20, 1589-1598

Libicki S.B, Salmon P.M, Robertson C.R. (1988) *The effective diffusive permeability of a nonreacting solute in microbial aggregates.* Biotech. Bioeng, 32, 68-85

Matson J.V, Charaklis W.G. (1976) *Diffusion into microbial aggregates.* Water Res, 10, 877-885

McCarty P.L. (1964) *Anaerobic waste treatment fundamentals.* Public Works, 95(9), 107

Perry R.H, Green D. (1984) *Chemical Engineers' Handbook*, 6th ed, McGraw Hill, N.Y, 3-319

Scheller F.W, Schubert F, Renneberg R, Müller H.G, Jänchen M, Weise H. (1985) *Biosensors: trends and commercialization.* Biosensors, 1, 135-160

Shieh W.K, Sutton P.M, Kos P. (1981) *Predicting reactor biomass concentration in a fluidized-bed system.* J. Water Poll. Contr. Fed. 53, 1574-1584

Speece R.E. (1988) *A survey of municipal anaerobic sludge digesters and diagnostic activity assays.* Wat. Res, 22, 365-372

Tanaka H, Matsumura M, Veliky I.A. (1984) *Diffusion characteristics of substrates in Ca-alginate gel beads.* Biotech. Bioeng, 26, 53-58

Van den Berg L, Patel G.B, Clark D.S, Lentz C.P. (1975) *Factors affecting rate of methane formation from acetic acid by enriched methanogenic cultures.* Can. J. Microbiol, 22, 1312-1319

Van den Heuvel J.C. (1992) *Mass transfer in and around biofims.* In: Biofilms - Science and Technology (L. Melo et al, eds.) Kluwer, Dordrecht, The Netherlands

Weber H, Kulbe K, Chmiel H.D, Trosch W. (1984) *Microbial acetate conversion to methane: kinetics, yields and pathways in a two-step digestion process.* Appl. Microbiol. Biotechnol, 19, 224-228

Yang S.T, Okos M.R. (1987) *Kinetic study and mathematical modelling of methanogenesis of acetate using pure cultures of methanogens.* Biotechnol. Bioeng, 30, 661-667

Yao T. (1983) *A chemically-modified enzyme membrane electrode as an amperometric glucose sensor.* Anal. Chim. Acta, 148, 27-33

INDUSTRIAL MONITORING: HYGIENE IN FOOD PROCESSING

J.T. HOLAH
Campden Food and Drink Research Association
Chipping Campden
Glos GL55 6LD
United Kingdom

1. Introduction

Cleaning and disinfection, together known as sanitation, is undertaken to remove all undesirable material (food residues, microorganisms, foreign bodies and cleaning chemicals) from surfaces, to a level such that residues remaining are of minimal risk to the safety or quality of the product. Sanitation is, therefore, the major day to day control of the 'surface' factor of the environmental routes of food product contamination. When undertaken correctly, sanitation programmes are cost effective, easy to manage and can reduce the risk of microbial or foreign body contamination. This will become increasingly pertinent in the future given the intrinsic demand for higher standards of hygiene in the production of short shelf-life chilled foods, together with pressure from customers, consumers and legislation for ever increasing hygiene standards. Sanitation demands, therefore, the same degree of attention as any other key process in the manufacture of safe and wholesome food products.

Due to its role in the control of environmental routes of product contamination and as with other important aspects of safe and wholesome food production, sanitation programmes require regular evaluation of their effectiveness as part of a structured QA system. Part of such a QA system is an audit of the sanitation programme, a procedure which usually takes into account (a) a visual and/or practical examination (typically by microbiological sampling) to assess for compliance with sanitation schedules, (b) an investigation into documented sanitation records and (c) evidence of any remedial action undertaken if failings in the programme were observed. This article deals with compliance with the sanitation schedule only, and most comments are aimed at sanitation programmes concerned with cleaning and disinfection to the highest standards e.g. 'high care' areas in the chilled food industry.

Within the environmental routes, and especially for surfaces, it is now realised that disinfection plays a major role in the control of contamination and thus influences the safety of the food product. The role of surface attachment of microorganisms in relation to disinfection resistance is becoming apparent and the influence of this phenomena and its affect on the design of disinfectant test procedures is detailed.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 645–659.

2. Sanitation schedule

A sanitation schedule details the sanitation programme and must show clearly, for all equipment and areas in the processing environment, each stage of the cleaning and disinfection process; (a) all pertinent information on chemicals, methods and safety (b), operatives and management responsibilities and (c) the key inspection points and how these should be inspected. Sanitation procedures are concerned with both the stages in which the sanitation programme is implemented and the sequence in which equipment and environmental surfaces are cleaned and disinfected within the processing area. The principle stages of the sanitation programme are detailed below.

2.1 PRODUCTION PERIODS

To facilitate a smooth and efficient sanitation programme, production operatives should be encouraged to undertake (a), product removal from lines during break periods (b), good housekeeping practices and (c), sound sanitation practices to clean up large product spillages during production. It is important, therefore, to also audit during production and in particular, attention should be paid to whether sanitation practices could affect the safety and quality of the product.

The potential for cleaning equipment to produce viable bacterial aerosols from test surfaces contaminated with attached biofilms, and thus disperse microbial contamination, has been demonstrated (Holah *et al.* 1990). Results showed that high pressure low volume (HPLV) and low pressure high volume (LPHV) techniques disperse a significant level of aerosol to a height greater than a typical food contact surface (1m) and should not, therefore, be used during production periods in 'high risk' food processing areas. Other techniques such as floor scrubbers, automats and shrouded HPLV lances, however, are acceptable for use in clean-as-you-go operations (which are to be encouraged) as the chance of contamination to product is low. After production HPLV and LPHV techniques may be safely used (and are likely to be the appropriate choice) but it is required that disinfection of food contact surfaces is the last operation to be performed within the sanitation programme.

2.2 PREPARATION

As soon as possible after production, equipment should be dismantled as far as is practicable or necessary and unwanted utensils/equipment removed. Dismantled equipment should be stored on racks or tables, not on the floor. Machinery should be switched off and electrical and other sensitive systems protected from water/chemical ingress. The preparation stage should, therefore, ensure that the sanitation programme can achieve its aims by reaching all surfaces and that the programme has no unavoidable deleterious affects on production equipment.

2.3 GROSS SOIL REMOVAL

Where appropriate, all loosely adhered or gross soil should be removed by e.g. brushing, scraping, shovelling or vacuum i.e. not by washing to drains using hoses or by compressed air.

2.4 PRE-RINSE

Surfaces should be rinsed with low pressure cold water to remove loosely

adhered small debris. Hot water can be used for fatty soils but too high a temperature may coagulate proteins.

2.5 CLEANING

Within the sanitation programme, the cleaning phase can be divided up into three sections, following the work of Jennings (1965) and interpreted by Koopal (1985), and is described below:

1. The wetting and penetration by the cleaning solution of both the soil and the equipment surface.

2. The reaction of the cleaning solution with both the soil and the surface to facilitate: peptization of organic materials, dissolution of soluble organics and minerals, emulsification of fats and the dispersion and removal from the surface of solid soil components.

3. The prevention of redeposition of the dispersed soil back onto the cleansed surface.

To undertake a sanitation programme, therefore, energy must be added to the soil to reduce both soil particle-soil particle and soil particle- equipment surface interactions. The mechanics and kinetics of these interactions have been discussed by a number of authors (Jennings 1965, Schlussler 1975, Loncin 1977, Corrieu 1981, Koopal 1985 and Bergman & Tragardh 1990) and fall beyond the scope of this article. In practical terms, however, sanitation programmes employ a combination of four major factors; mechanical or kinetic energy, chemical energy, temperature or thermal energy and time.

Mechanical or kinetic energy is used to physically remove soils and may include scraping, manual brushing and automated scrubbing (physical abrasion) and pressure jet washing (fluid abrasion). Chemical energy is used to break down soils to render them easier to remove and to suspend them in solution to aid rinsability. Chemical effects increase with temperature linearly and approximately double for every 10°C rise and for fatty and oily soils, temperatures above their melting point are used to break down and emulsify these deposits and so aid removal. For cleaning processes using mechanical, chemical and thermal energies, generally the longer the time period employed, the more efficient the process.

Cleaning is responsible for not only the removal of the soil but also the majority of the microorganisms present. Mrozek (1982) showed a reduction in bacterial numbers on surfaces by up to 3 log orders, Schmidt and Cremling (1981) described reductions of 2-6 log orders, whilst swabbing analysis of chilled food processing equipment suggests that greater than 4 log orders may be obtained by cleaning, with an additional 1 log order reduction by the disinfection phase (Holah *in press*). Emphasis should be placed, therefore, on the cleaning stage of the sanitation programme and a good quality detergent should be used.

A cleaning solution or detergent is blended form a range of typical components including water, surfactants, inorganic alkalis, inorganic and organic acids and sequestering agents, though unfortunately, no one cleaning agent is able to perform all the functions necessary to facilitate a successful cleaning programme. A number of products may be required, therefore, but this

requirement must be balanced by the desire to keep the range of cleaning chemicals on site to a minimum so as to reduce the risk of using the wrong product, to simplify the job of the safety officer and to allow chemical purchase to be made more on the economics of bulk quantities. The range of chemicals and their purpose is well documented (Anon 1991, Elliot 1980, ICMSF 1980, 1988, Hayes 1985, Holah 1991, Koopal 1985, Russell *et al.* 1982).

The methodology used in the sanitation programme must be capable of achieving the required results and is selected dependant on the size of the area to be cleaned and the degree and type of soiling to be removed. The use of cleaning techniques can perhaps be best described schematically following the information detailed in Figure 1 (modified from Offiler 1990) which shows the different energy source inputs for a number of cleaning techniques and shows their ability to cope with both low and high (dotted line) levels of soiling. In small food operations manual cleaning is generally used as it can apply high levels of mechanical energy in a well directed manner, though for larger food processing operations, gels or foam are used to obtain sufficient chemical contact time and are rinsed away with water pressure.

Cleaning equipment is prone to contamination with *Listeria* spp and other pathogenic microorganisms and by the nature of its use, provides an excellent way in which contamination can be transferred from area to area. Sanitation equipment should be hygienically designed e.g. brushes should have the head and bristles moulded as one unit or alternatively, have bristles of coloured, impervious material e.g. nylon, embedded into the head with resin so no soil trap points are apparent. After use, cleaning equipment should be thoroughly cleaned and if appropriate, disinfected.

2.6 INTER-RINSE

Both soil detached by cleaning operations and cleaning chemical residues should be removed from surfaces by rinsing with low pressure cold water.

2.7 DISINFECTION

Although the majority of microbial contamination is removed by the cleaning phase of the sanitation programme, for many food processing operations there are likely to be sufficient viable microorganisms remaining on the surface to warrant the application of a disinfectant. The aim of disinfection is, therefore, to further reduce the surface population of viable microorganisms, via removal or destruction, and/or to prevent surface microbial growth during the inter-production period.

The efficiency of disinfectants is generally controlled by four factors; interfering substances (primarily organic matter), pH, concentration and contact time. To some extent, and particularly for the oxidative biocides, the efficiency of all disinfectants is reduced in the presence of organic matter. Organic material may react chemically with the disinfectant such that it loses its biocidal potency or spatially such that microorganisms are protected from its effect. Surfaces should, therefore, be thoroughly clean before disinfectants are applied. Disinfectants should only be used within the pH range as specified by the manufacturer as outside this range they may have no activity. In practice the relationship between microbial death and disinfectant concentration is not linear but follows a sigmoidal curve. It is important, therefore to use the disinfectant

at the concentration as recommended by the manufacturer and be aware that changes to this concentration may not enhance effects as desired. Sufficient contact time between the disinfectant and the microorganisms is perhaps the most important factor controlling biocidal efficiency. Most general purpose disinfectants are formulated to require at least 5 minutes to reduce bacterial populations by 5 log orders in suspension. For particularly resistant organisms such as spores or moulds, surfaces should be repeatedly dosed to ensure contact time for 15-60 minutes.

Disinfectants used in food processing are generally restricted as toxicity and taint potential are as important as their ability to reduce microorganism viability. Typical disinfectants used within the food industry include chlorine, quaternary ammonium compounds (QUATS), amphoterics, iodophors, peracetic acid, glutaraldehyde and biguanides.

2.8 POST-RINSE

Disinfectant residues should be removed by rinsing away with low pressure cold water of known potable quality. Some disinfectants, however, are intended to be left on surfaces until the start of subsequent production periods and should be formulated to be both surface active and of low risk, in terms of taint or toxicity, to foodstuffs. Potential EEC legislation could dictate that all disinfectants are to be rinsed.

2.9 SEQUENCE

A sanitation sequence determines the order in which the product contact (equipment) and environmental surfaces (walls, floors, drains etc.) are sanitised such that once product contact surfaces are disinfected, they are not recontaminated. A typical sanitation sequence would be:

1. Remove gross soil from production equipment.
2. Remove gross soil from environmental surfaces.
3. Rinse down environmental surfaces.
4. Rinse down equipment and flush to drain.
5. Clean environmental surfaces.
6. Rinse environmental surfaces.
7. Clean equipment.
8. Rinse equipment.
9. Disinfect equipment.
10. Fog.

2.10 INTER-PRODUCTION CYCLE CONDITIONS

Procedures should be undertaken to prevent the growth of microorganisms on production contact surfaces, or surface recontamination, in the period up until the next production process.

2.11 PERIODIC PRACTICES

Soil removal from surfaces follows first order reaction kinetics such that the log of the mass of soil per unit area remaining is linear with respect to cleaning time (Jennings 1965, Schlusser 1975). Routine cleaning operations are, therefore, never 100% efficient and over a course of soiling/cleaning cycles, soil deposits (potentially including microorganisms) will be retained. As soil accumulates, cleaning efficiency will decrease and soil deposits may for a period grow exponentially. The time scale for such soil accumulation will differ for all processing applications and can range from hours (e.g. heat exchangers) to weeks and in practice is controlled by the application of a 'periodic' clean (Dunsmore *et al.* 1981). A periodic clean is used to bring soiling levels down to a baseline and involves the input of greater cleaning energy or the dismantling of the equipment etc.

Periodic practices also incorporate the undertaking of longer term sanitation practices e.g. sanitation of surfaces, fixtures and fittings above 2m or cleaning and disinfection of chillers.

3. Performance Assessments

The performance of sanitation programmes designed for open surface cleaning is undertaken on two levels, an immediate assessment by sensory evaluation and an historical measurement of the performance of the sanitation programme (if the sensory evaluation is satisfactory) by an enumeration of remaining microbial surface populations. Sensory evaluations are used as a process control to immediately rectify grossly poor sanitation whilst microbiological assessments may be used to ensure compliance with microbial standards, in hygiene inspection and troubleshooting exercises, to optimise sanitation procedures and as a basis for cleaning operatives bonus payments.

Rapid methods have been recently developed to assess microbial surface populations and/or soil residuals in a time relevant to process control, usually taken as less than 15-20 minutes. As such, this has led to the ability to provide the same information as the combined traditional sensory and historical evaluation and thus lead to the correction of both sensory and microbiologically poor sanitation practices in a time scale such that the sanitation programme can be corrected.

In the same way that traditional food 'end product analysis' only samples a fraction of the total production batch, surface hygiene assessment techniques only sample a very small proportion of production plant surfaces. As with modern food production practices, hygiene assessment techniques should, therefore, monitor the effectiveness of a structured Hazard Analysis Critical Control Point (HACCP) approach. Common CCP's include; detergent and disinfection concentrations, chemical solution temperatures, chemical contact times, degree and time of application procedures, cleaning equipment settings (e.g. water pressure and nozzle type or orbital speed and scrubbing brush type) degree of kinetic or mechanical inputs, cleaning equipment maintenance and chemical stock rotation.

3.1 METHODOLOGY

Sensory evaluation involves visual inspection of surfaces under good lighting, smelling for product or offensive odours and feeling for the build up of product residues. If sensory assessments indicate the presence of product residues, further assessments are not required as quite clearly, the sanitation programme has not been performed to the required standard. Microbiological examination could also be misleading.

If no product residues are detected, microbiological (or chemical) techniques may be applied. Traditional microbiological techniques appropriate for food factory use have been extensively reviewed (Patterson 1971, Baldock 1974, Kulkarni *et al.* 1975) and involve the removal or sampling of microorganisms from surfaces and their culture using standard microbiological methods. Microorganisms may be sampled via sterile cotton or alginate swabs and sponges, after which the microorganisms are resuspended by vortex mixing or dissolution into suitable recovery or transport media. For larger enclosed areas (e.g. fillers) surfaces may be sampled using rinse solutions. Representative dilutions are then incubated in a range of microbiological growth media, dependant on which microorganisms types are being selected for, and incubated for 24-48 hours. Alternatively, microorganisms may be sampled directly onto self prepared or commercial ('dip slides') agar contact plates. A description of a range of infrequently used chemical tests to assess soil retention on surfaces is given in the review of Corrieu (1981).

Rapid methods are now becoming more readily used for hygiene assessment and the two most common techniques are epifluorescent microscopy and ATP. With epifluorescent microscopy, microorganisms are first sampled by swabbing or rinsing and then collected onto special filters by filtering the swab resuspension fluid (Holah *et al.*, 1989) or the rinse liquid (McKinnon and Mansell, 1981). Microorganisms retained on the filters are enumerated using a modified direct epifluorescent filter technique (DEFT) method of Pettipher *et al.* (1981). Alternatively, microorganisms may by enumerated directly on the surface of coupons attached to product contact surfaces by direct epifluorescent microscopy (DEM) as described by Holah *et al.* (1988). Both DEFT and DEM techniques can be completed in around 20 min.

The ATP technique is based on the assessment of levels of adenosine triphosphate (ATP), present in animal, plant and microbial cells, via an enzyme linked system which produces light in proportion to the concentration of ATP present. Surfaces are sampled by swabbing or rinsing (Thompson 1989, Simpson 1989) and upon addition of suitable reagents, the level of ATP can be measured in a luminometer in approximately 5 min. In the majority of practical applications, where an assessment of cleaning is required, analysis for total ATP (i.e. from both microorganism and product residues) is preferred on the assumption that any residues, soil or microorganisms, should have been removed. ATP techniques have the additional benefit as luminometers can now be obtained as portable, battery operated units that can be used at point of sampling. Both DEFT and DEM, however, require the use of laboratory facilities.

3.2 PERFORMANCE AND ACCURACY

The 'acceptable' number of microorganisms remaining on a surface after

sanitation is dependant on the food product, process, 'risk area' and degree of sanitation undertaken. Figures quoted include (as total viable count per square decimetre) 100 (Favero *et al.* 1984), 540 (Thorpe and Baker (1987) and 1000 (Timperley and Lawson (1980) for dairies, canneries and general manufacturing respectively, whilst for chilled food production, sanitation programmes should achieve levels of around 1000 microorganisms per swab (Holah *in press*). Expressing counts arithmetically is always a problem, however, as single counts taken in areas where cleaning has been inadequate (which may be in excess of 10^8 per swab) produce an artificially high mean count, even over thousands of samples. It is better, therefore, to express counts as log to the base 10, a technique which places less emphasis on a relatively few high counts, and Holah (*in press*) has reported that log counts of 1 or less per swab can be obtained in chilled food production areas.

In terms of accuracy, DEM is the most accurate of the surface hygiene assessment techniques (Holah 1990, 1991), whilst traditional swabbing, contact plates, ATP and DEFT are equally precise. These techniques are only accurate (as compared to DEM) for surface populations above 10^6 bacteria/cm^2 and for populations of approximately 10^4 bacteria/cm^2, single population estimates have an accuracy of approximately +/- 2 log orders. Single surface hygiene assessments, i.e. as undertaken for inspection purposes, are only accurately indicative of gross surface contamination. For samples taken on a routine basis as part of a QA/QC programme, results which are predominantly 'low' indicate a sanitation system in control whilst for results which are variable, variation is as likely due to sampling errors as sanitation system deficiencies.

For enclosed food production equipment, the assessment of surface hygiene levels is particularly difficult. If surfaces are accessible, sensory, traditional and rapid techniques may be used but for inaccessible surfaces, reliance is placed on related parameters such as cleaning solution conductivity or clarity, a knowledge of the cleaning parameters used (chemicals, temperatures, circulation times and velocities) as established on commissioning by strip down procedures and the microbiological analysis of the first batch of product processed. Currently within Europe, a FLAIR Project 'Sanitation of food Processing Plants' (No. 89082) has an objective to develop on-line sensors which can detect films and deposits on the internal surfaces of liquid handling processing equipment (e.g. pipes, bends, plate heat exchangers) and which can be connected to a PC-based process control system. The sensors are intended to detect when films have reached a thickness such that they compromise production efficiency and also the absence of deposits to establish whether sanitation has been undertaken effectively.

Sensor methodology investigated to date include ultrasonics for pipework, vibrational analysis for plate heat exchangers and optical devices in conjunction with sightglasses. In principle, all have proved adequate for the measurement of film thicknesses to a maximum of 2mm and will be further studied. Results of the FLAIR Project remain confidential to the participants for a short term after the project is completed but it is hoped that this work will enhance the safety and quality of food production by allowing food processors to be immediately aware of the hygienic quality of internal equipment surfaces.

4. Disinfection Studies

When disinfectants are assessed for their use in food hygiene, they are usually

evaluated in a variety of suspension tests and such tests have been comprehensively reviewed (Anon 1981, Reybrouk 1982). In practice, however, microorganisms that are disinfected on food production surfaces are those that are remaining after the cleaning stage and are thus likely to be surface attached.

TABLE 1. Comparison of disinfectant perfomance against bacteria in suspension and attached to surfaces

		SUSPENSION TEST			SURFACE TEST	
PRODUCT	CONC (%)	0.03	1.0	CONC (%)	0.03	1.0
Peracid	0.1	P	P	0.1	F	F
				1.0	P	P
				10.0	P	P
Peracid	0.1	P	P	0.1	F	F
				1.0	P	P
				10.0	P	P
Iodophore	0.2	P	F	0.2	F	F
				2.0	P	P
				20.0	P	P
Iodophore	1.0	F	F	1.0	F	F
				10.0	P	P
Amphoteric	1.0	P	P	1.0	F	F
				10.0	P	P
Amphoteric	1.0	P	P	1.0	F	F
				10.0	P	F
Biguanide	0.5	P	F	0.5	F	F
				5.0	P	F
				50.0	P	P
Biguanide	0.5	P	F	0.5	F	F
				5.0	F	F
				50.0	P	F
QUAT	1.0	P	P	1.0	F	F
				10.0	F	F
QUAT	1.0	P	P	1.0	F	F
				10.0	F	F
HOCl	0.25	P	F	0.25	F	F
				2.5	F	F
				25.0	P	P
HOCl	0.3	F	F	0.6	F	F
				6.0	F	F
				30.0	P	P

4.1 DISINFECTANT RESISTANCE

A number of authors have shown that bacteria attached to various surfaces are

more resistant to biocides than when the organisms are in suspension: suspended particulate matter (Ridgway and Olsen 1982), glass (Hugo et al.. 1985, Frank and Koffi 1990) and Douglas fir and PVC (Wright *et al.* 1991). Specifically, bacteria grown on the surfaces of granular activated carbon particles, metal coupons and glass microscope slides were shown to be 150 to more than 3000 times more resistant to hypochlorous acid (HOCl) than were unattached cells (LeChevallier et al.. 1988) and oral bacteria growing in biofilms were shown to be ten thousand times more resistant to chlorhexidine than when in the planktonic phase. In addition, Lee and Frank (1991) demonstrated that resistance increased with biofilm age.

4.2 USE OF NEW TECHNOLOGIES

Surface based test methodologies are available for assessing the efficacy of food industry based disinfectants, though in each case bacteria are dried onto carrier surfaces (Anon. 1967, 1984, 1986). To evaluate disinfectants against microorganisms in biofilms, impediametric techniques have been developed (Holah *et al.* 1990). Results, for a range of 12 commonly used disinfectants, were compared to those achieved by the current suspension test method of choice, the "European Suspension Test" (EST)(Anon. 1987). When compared to test microorganisms in suspension using the EST, test microorganisms attached to stainless steel (Table 1) required 10-100 times the manufactures recommended in-use concentration to obtain the same viability reduction. In addition, disinfectants that gave good viability reductions in the EST did not necessarily give similar performances against attached microorganisms and vice versa. This suggests that traditionally used biocides may not be as suitable for surface disinfection as supposed and that new testing methodologies may lead to the development of more effective products. These methodologies may involve impediametric measurements using Bactometer, Malthus or Don Whitley instruments or novel techniques such as the application of *in vivo* bioluminescence using *lux AB* recombinant derivatives (Walker *et al.*. 1992). The use of bioluminescence also has the advantage that results can be obtained in a time related to process control in industry.

The enhanced biocidal resistance of attached bacteria is probably related to their environment. Three differences between the environments of attached and free-living bacteria were described by Fletcher (1974); adsorption of nutrients, cell envelope interactions and the formation of a polymeric matrix. A solid/liquid interface is thermodynamically unstable and solutes may be adsorbed to the surface. An adsorption of organic matter may thus form a protective layer surrounding attached bacteria. Deformation of the cell envelope may occur due to the attractive forces which hold the cell at the surface. Since all disinfectants must bind to and then traverse the cell envelope to reach their target sites (Klemperer 1982), any modification of the cell envelope is likely to affect disinfection efficiency. During biofilm formation bacteria may produce extracellular polymers which tend to form a polymeric matrix in which the bacteria become embedded. The presence of this matrix may prevent the penetration of disinfectants to the embedded cells. The importance of the topography of the surface to bacterial removal has been demonstrated by Holah & Thorpe (1990). Bacteria attached in pits and crevices were very difficult to remove by cleaning agents, because of poor chemical penetration. Such difficulties, associated possibly with surface tension, may also hinder the

penetration of disinfectants.

In reality, however, many factors are present in the factory environment which may affect the resistance of bacteria prior to disinfection. These are chiefly related to the total sanitation programme and include the action of detergents, high temperatures, pH and mechanical stress, as well as attachment. To examine the effect of these factors on disinfection resistance, the use of impediametric techniques are extremely beneficial as they allow the assessment of viability whilst attached organisms remain surface bound and thus maintain the physiological condition of the bacteria and alleviate the problems associated with bacterial removal from surfaces (Anon. 1986). Table 2 shows how the effects of a number of parameters, in this case temperature and agitation, can synergistically enhance the performance of disinfectants against attached microorganisms. In the future, multi-parameter studies may aid food processors select optimum conditions under which disinfectants can be used.

4.3 DISINFECTANT TESTING IN EUROPE

With the legislative procedures currently taking place in Europe with respect to free trade, efforts are being made to harmonise disinfectant test procedures through CEN/ TC 216. It is the intention of this committee to structure test methodologies into three phases. Phase 1 will contain basic suspension test methodologies to assess for bactericidal, fungicidal, sporicidal (possibly) and virucidal (possibly) properties. Phase 2 will be divided into three working groups which will cover the medical, veterinary and food hygiene (including domestic and institutional) fields respectively. Each group will develop suspension and surface tests to assess bactericidal, fungicidal, sporicidal and virucidal efficacy under 'practical' conditions pertinent to their field areas. Phase 3, associated with the working groups, will develop test methodologies for 'naturally' contaminated surfaces, if possible and if necessary.

TABLE 2. The effect of temperature and agitation on disinfection of surface attached bacteria

	TEMPERATURE ^{o}C							
	4		22		37		58	
	P.a	S.a	P.a	S.a	P.a	S.a	P.a	S.a
Control (Mean log number)	6.64	6.36	6.49	6.29	6.5	6.4	4.19	4.67
After treatment								
Biocide only	6.3	6.08	5.46	5.45	5.07	4.67	4.68	3.45
Agitation/biocide	5.58	4.96	4.98	4.97	4.97	4.67	4.13	3.05
Mean log reduction								
Biocide only	0.14	0.28	1.03	0.84	1.43	1.73	-0.49	1.22
Agitation/biocide	1.06	1.4	1.51	1.32	1.53	1.73	0.06	1.62

Work is progressing smoothly with relation to traditional suspension tests and CEN Standards are expected in 1993, though the use of surface tests has been 'questioned' by disinfectant manufacturers. Difficulties arise in the selection of surface test for two main reasons. Firstly, manufactures are generally legally required to detail an in-use concentration on the product label and have traditionally done so on the basis of suspension test results. Quite clearly, results from surface tests may indicate higher required concentrations and/or show differences in efficacy. Secondly, interpretation of the requirement of surfaces tests may be difficult as chemical manufactures claim that the mechanical action used when disinfectants are applied by brushing or wiping etc. results in surface bound organisms being brought into suspension and thus suspension test are more appropriate. For the vast majority of food industry disinfectant applications, however, mechanical energy is not used as the biocide is applied by misting surfaces or immersion. To ensure that the food industry obtains the disinfectant performance information it clearly needs, it is hoped that CEN/ TC 216 will adopt sensible test methodologies, based on the continuing evidence that demonstrates the influence on disinfection resistance of surface attachment, that involve the use of surface test procedures.

References

Anon. (1967) Tube test for the evaluation of detergent-disinfectants for dairy equipment. *International Standard FIL-IDF44,* International Dairy Federation, Brussels.

Anon. (1984) AOAC Use-Dilution Method. In *Official Methods Of Analysis of the Association of Official Chemists,* 14th Edition, ed S.Williams. Association of Official Analytical Chemists, Inc., Arlington.

Anon. (1986) *AFNOR T72-190 Germ-carrier method.* l'Association Francaise de Normalisation, Paris.

Anon. (1987) *Method of test for the antimicrobial activity of disinfectants in food hygiene.* Strasbourg: Council of Europe.

Anon. (1981) *Disinfectants. Their use and evaluation of effectiveness.* Eds. C.H. Collins, M.C. Allwood, S.F. Bloomfield and A. Fox, Society for Applied Bacteriology Technical Series No. 16, Academic Press, London.

Anon (1991) Chapter 5, 'Sanitation'. In *Principles and Practices for the Safe Processing of Foods.* Eds. D.A. and N.F. Shapton, Butterworth-Heinemann Ltd, Oxford.

Baldock, J.D. (1974) Microbiological monitoring of the food plant: methods to assess bacterial contamination on surfaces. *Journal of Milk Food Technology* 37, 361-368

Bergman, B-O. and Tragardh, C. (1990) An approach to study and model the hydrodynamic cleaning effect. *Journal of Food Process Engineering* 13, 135-154

Corrieu, G. (1981) State-of-the-art of cleaning surfaces. In Proceedings of *Fundamentals and Application of Surface Phenomena Associated with Fouling and Cleaning in Food Processing.* April 6-9, Lund University, Sweden.

Dunsmore, D.G., Twomey, A., Whittlestone, W.G. and Morgan, H.W. (1981) Design and performance of systems for cleaning product contact surfaces of food equipment: A review. *Journal of Food Protection* 44, 220-240, 1981.

Elliot, R.P. (1980) Cleaning and sanitation. In *Principals of food processing sanitation.* Ed Katsuyama, A.M., The Food Processors Institute, U.S.A.

Favero, M.S., Gabis, D.A. and Veseley, D. (1984) Environmental monitoring procedures. In *Compendium of Methods for the Microbiological Examination of Foods.* Ed. M.L. Speck, American Public Health Association, Washington, DC.

Fletcher, M. (1984) Comparative physiology of attached and free-living bacteria. In *Microbial Adhesion and Aggregation,* Ed Marshall,K.C., Springer-Verlag, Berlin.

Frank, J.F. and Koffi, R.A. 1990 Surface-adherant growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitisers and heat. *Journal of Food Protection* 53, 550-554

Hayes, P.R. (1985) Chapter 9, Cleaning and Disinfection: Methods. In *Food Microbiology and Hygiene,* Elsevier Applied Science Publishers, London.

Holah, J.T. (1990) Monitoring the hygienic status of surfaces. In Proceedings of *'Hygiene for the 90's'.* Campden Food and Drink Research Association, Chipping Campden, UK.

Holah, J.T. (1991) Food Surface Sanitation. In *Encyclopedia of Food Science and Technology.* Ed. Y.H. Hui, John Wiley and Sons, New York.

Holah, J.T. (In press) Cleaning and Disinfection. In *Chilled Foods: A Comprehensive Guide.* Eds C. Dennis and M.F. Stringer, Ellis Horwood , Chichester.

Holah, J.T., Betts, R.P. and Thorpe, R.H. (1988) The use of direct epifluorescent microscopy (DEM) and the direct epifluorescent filter technique (DEFT) to assess microbial populations on food contact surfaces. *Journal of Applied Bacteriology* 65,215-221

Holah,J.T., Betts,R.P. and Thorpe,R.H. (1989) The use of epifluorescence microscopy to determine surface hygiene. *International Biodeterioration* 25, 147-153

Holah, J.T., Higgs, C., Robinson, S., Worthington, D. and Spenceley, H. (1990) A Malthus Based Surface Disinfection Test For Food Hygiene. *Letters in Applied Microbiology* 11, 255-259

Holah, J.T. and Thorpe, R.H. (1990) Cleanability in relation to bacterial retention on unused and abraded domestic sink materials. *Journal of Applied Bacteriology* 69, 599-608

Holah, J.T., Timperley, A.W. and Holder, J.S. (1990) *The spread of Listeria by cleaning systems.* Technical Memorandum No. 590, Campden Food and Drink Research Association, Chipping Campden, UK.

Hugo, W.B., Pallent, L.J., Grant, D.J.W., Denyer, S.P. and Davies, A. (1985) Factors contributing to the survival of *Pseudomonas cepacia* in chlorhexidine. *Letters in Applied Microbiology* 2, 37-42

ICMFS (1980) Chapter 14; Cleaning, Disinfection and Hygiene, pp232-258. In *Microbial Ecology of Foods, Volume 1, Factors Affecting Life and Death of Microorganisms.* The International Commission on Microbiological Specifications for Foods, Academic Press, London.

ICMFS (1988) Chapter 6; Cleaning and disinfecting, pp93-116. In *Microorganisms in Foods, Volume 4, Application of the Hazard Analysis Critical Control Point (HACCP) System to Ensure Microbiological Safety and Quality.* The International Commission on Microbiological Specifications for Foods, Blackwell Scientific, Oxford.

Jennings, W.G. (1965) Theory and practice of hard-surface cleaning. *Advances in Food Research* 14, 325-459, 1965.

Keevil, C.W. and Mackerness, C.W. 1990 Biocide treatment of biofilms.

International Biodeterioration 26, 169-179
Klemperer, R. (1982) Tests for disinfectants: principals and problems. In *Disinfectants: their assessment and industrial use*, Scientific Symposia Ltd, London.
Koopal, L.K. (1985) Physico-chemical aspects of hard-surface cleaning.*Netherlands Milk and Dairy Journal* 39, 127-154
Kulkarni, S.M., Maxy, R.B. and Arnold, R.G. (1975) Evaluation of soil deposition and removal processes: An interpretive view. *Journal of Dairy Science* 58, 1922-1936
Lechevallier, M.W., Cawthorn, C.D. and Lee, R.G. (1988) Inactivation of biofilm bacteria. *Applied and Environmental Microbiology* 54, 2492-2499
Lee, S-H. and Frank, J.F. 1991 Inactivation of surface-adherant *Listeria monocytogenes*: Hypochlorite and heat. *Journal of Food Protection* 54, 4-6
Loncin, M. (1977) Modelling in cleaning, disinfection and rinsing. In proceedings of "*Mathematical modelling in food processing*", 7-9 September, Lund. Lund Institute of Technology, 1977.
Mckinnon, C.M. and Mansell, R. (1981) Rapid counting of bacteria in rinses of milking equipment by a membrane filtration epifluorescent filter technique. *Journal of Applied Biology* 51, 363-369
Mrozek, H. (1982) Development trends with disinfection in the food industry. *Deutsche Molkerei-Zeitung* 12, 348-352
Offiler,M.T. (1990) Open plant cleaning: equipment and methods. In proceedings of '*Hygiene for the 90's*', November 7-8, Campden Food and Drink Research Association, Chipping Campden, Glos., U.K.
Patterson, J.T. (1971) Microbiological assessment of surfaces. *Journal of Food Technology* 6, 63-72
Pettipher, G.L., Mansell, R. and Mckinnon, C.M. (1980) Rapid membrane filtration epifluorescent microscopy technique for direct enumeration of bacteria in raw milk. *Applied and Environmental Microbiology* 39, 423-429
Raybrouk, G. 1982 The evaluation of the antimicrobial activity of disinfectants. In *Principles and Practice of Disinfection*, Eds. A.D. Russell, W.B. Hugo and G.A.J. Ayliffe, Blackwell Scientific Publications, London.
Ridgway, H.F and Olsen, B.H (1982) Chlorine resistance patterns of bacteria from two drinking water distribution systems. *Applied and Environmental Microbiology*, 44, 972-987
Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. (1982) *Principles and Practice of Disinfection*, Blackwell Scientific Publications, London.
Schmidt, U. and Cremmling, K. (1981) Cleaning and disinfection processes. IV. Effects of cleaning and other measures on surface bacterial flora. *Fleischwirtschaft* 61, 1202-1207
Schlussler, H.J. (1975) Zur Kinetik von Reinigungsvorgangen an festen Oberflachen. Symposium uber *Reinigen und Desinfizieren lebensmittelverarbeitender Anlagen.* Karlsruhe, 1975.
Simpson, W.J. (1989) "Instant" assessment of brewery hygiene using ATP Bioluminescence. *Brewers Guardian* 118, 20-22
Thompson, P. (1989) Rapid hygiene analysis using ATP Bioluminescence. *European Food and Drink Review*, Spring Issue, 42-48
Thorpe, R.H. and Barker, P.M. (1987) *Hygienic Design of Liquid Handling Equipment for the Food Industry.* Technical Manual No. 17, Campden Food and Drink Research Association, Chipping Campden, UK.
Timperley, D.A. and Lawson, G.B. (1980) Test rigs for evaluation of hygiene in

plant design. In *Hygienic Design and Operation of Food Plant.* Ed. R. Jowitt, Ellis Horwood, Chichester.

Walker, A.J., Jassim, S.A.A., Holah, J.T., Denyer, S.P. and Stewart, G.S.A.B. (1992) Bioluminescent *Listeria monocytogenes* provide a rapid assay for measuring biocide efficacy. *FEMS Microbiology Letters* 91, 251-256

Wright, J.B., Ruseska, I. and Costerton, J.W. (1991) Decreased biocide susceptibility of adherent *Legionella pneumophila. Journal of Applied Bacteriology* 71, 531-538

INDUSTRIAL MONITORING - COOLING WATER SYSTEMS

T.R. BOTT
School of Chemical Engineering
University of Birmingham
Edgbaston
Birmingham B15 2TT. UK

1. Introduction

In any industrial process it is necessary to maintain efficiency so that production costs are kept to a minimum and the profits of the organisation are made as high as possible. Earlier discussions have demonstrated the problems associated with the accumulation of biofilms on surfaces, and the techniques that are available to reduce or overcome the associated operating problems.

All cooling water systems require continuous surveillance if their efficiency is to be maintained. Although closed recirculating systems are used industrially, the major requirements for water cooling (eg. power stations and chemical complexes) are generally met by open recirculating or "once through" systems. It is these latter circuits that will form the basis for the present discussion but clearly some of the techniques for monitoring will also apply to closed systems.

Much of the water used industrially is natural in origin, ie. from rivers, canals, lakes or bore holes, and as a consequence is likely to change in composition with time. Each system will require careful management and treatment programmes.

In the handling of large volumes of water, that may cause biofilm formation (biofouling) and other problems in the particular process plant, for instance the condensers on a power station, action is necessary to overcome the difficulties. Biocides or physical methods are generally employed to maintain satisfactory operating conditions. It will be necessary to monitor the effectiveness of the remedial techniques, not least to make sure that they are cost effective.

Cooling water systems operate at temperatures above ambient and the cooling duty raises the temperature still further. As a result the water temperature within an associated heat exchanger is very close to the optimum for maximum biofilm growth. It is in relation to the heat exchangers therefore that biofilm monitoring should be directed.

Every cooling water system is a unique combination of equipment, water quality, water chemistry, contaminants, blowdown and control techniques. Monitoring of the effectiveness of the treatment programme to combat the formation of biofilms will require the collection of a great deal of information. Often this is a painstaking task because of the complexity

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 661–669.

of the system and the variations encountered in operation not only of the cooling water system itself, but variations in the conditions in the plant of which the cooling water system represents the cold utility.

2. Cooling water quality

The following lists some of the aspects of water quality that affect its utility.

Raw water:

Mud and silt that will be dependent on climatic conditions, eg. rainfall, summer or winter.

Soluble iron.

Dissolved solids dependent on the soil through which the water has percolated.

Biological material (living or dead) ie. flora and fauna depending on the system.

Airborne scrubbed by the water in the cooling tower or spray pond:

Dust particles dependent on other local activities such as grinding plant, combustion or building demolition and wind direction.

Acid gases, eg. CO_2 and SO_2 from combustion operations dependent upon wind direction.

Monitoring is required to establish the efficiency of treatment programmes applied to maintain the quality of the water to reduce or eliminate operating problems. Treatment will involve:

Biocides or biostats to control microbial activity

Inhibitors and pH control to eliminate or control corrosion

Chemical additives to reduce or prevent scale formation

Agents to control particulate deposition

In addition dispersants and detergents may be added to assist one or more of the above treatments.

Fluctuations in the quality of the water and in the system may be anticipated from a number of causes:

Changes in the season and local temperature not only as it affects the water in the system but also the origin of the water.

Local pH that may be substantially different from the bulk pH.

The effects of cutbacks in cooling water flow for heat exchanger control, brought about by changes in cooling water temperature, reduced production or changes in process plant operation.

The effects of other treatments such as scale prevention or corrosion control.

Some systems become resistant to the use of a particular biocide.

There can be effects of corrosion control and scale prevention on the extent of biological activity. Tables 1 and 2 list some common corrosion and scale inhibitors and their possible effects on microbial activity.

3. Monitoring

The monitoring process will involve not only biofilms but the system in general in order that potential changes may be anticipated.

Monitoring can be considered to fall into two basic categories as suggested by Flemming (1991):

1. Direct that monitors the biofilm itself, eg. the number of cells, activity, etc.
2. Indirect that monitors the biofilm through its effect
   a) Physical measurements such as flow resistance across the biofilm or thermal resistance through the film
   b) Enzyme activity
   c) Degradation of metabolites
   d) Local changes in pH. The micro electrodes described elsewhere (van den Heuvel 1992) could be useful in this respect

TABLE 1. Common corrosion inhibitors and possible effects on biological activities

Chemical	Comment
Chromates	Toxic and definitely environmentally friendly
Nitrates	Aquatic plant nutrient
Silicates	Slow acting
Tannins	Biological nutrient
Orthopohosphate	Aquatic plant nutrient
Zinc salts	Toxic
Polyphosphates	Sludge forming aquatic plant nutrient

TABLE 2. Scale preventatives that might give rise to biological nutrients

Chemical	Action
Natural organic compounds and polyacrylates	Modify crystal habit
Polyphosphates, organo phosphorous compounds	Retard precipitation dispersants for crystallites in suspension

4. Cooling water assessment

Assessment can involve a number of different methods including system inspections, chemical and microbiological examinations of the cooling water, variations in operating data, and the effect of other treatment programmes applied to the system.

4.1 SYSTEM INSPECTION

System inspection involves a visual assessment of the biofouling problem. For this reason it is confined to open conduits and cooling towers or spray ponds. Despite these limitations however, visual observation based on experience can, provide good indications of the condition of the system and represents the first approach to monitoring. It cannot of course, be considered as an exclusive method and more sophisticated techniques are required if adequate control of biofilm formation is to be established. On the other hand visual assessment which is largely based on qualitative experience, may anticipate the rather time consuming laboratory techniques and may provide an opportunity for earlier remedial treatment that might otherwise be more extensive after the lapse of a further few days needed to carry out laboratory investigations. At the same time visual detection does suggest that the biofilm growth may well have progressed to an advanced stage.

Table 3 gives an indication of the appearance of microbial fouling and biofilm formation.

TABLE 3. Appearance of microbial fouling

Micro-organism	Appearance
Algae	Blue-green colour visible in cooling tower, ponds, and gulleys. May be filamentous.
Fungi	Generally evident on surfaces eg. on cooling tower packing (especially wood). May be filamentous.
Bacteria	Slimy often black or coloured deposits on surfaces. Increased pressure drop and reduced heat transfer efficiency.

4.2 MICROBIAL MONITORING

One of the principal features of cooling water systems is that it is unusual for complete sterilisation to be attempted, since this is not necessary and in any case would be prohibitive in terms of cost. Under these circumstances it is important to carry out regular laboratory tests to ensure that the microbial population is kept within certain acceptable limits. The level of micro-organisms within the circulating water is generally a good general indicator of the likely extent of biofilms on equipment surfaces, although this may not always be the case as there can be exceptions to this general premise (see later).

Sometimes only total counts are run to indicate overall microbial populations. In industrial recirculating cooling water loops more specific analysis for certain species may be required. Changes in total counts do not always indicate changes in fungi, algae, anaerobic bacteria, or sulphate reducers. Since these micro-organisms can cause different troubles for the plant operator they must be separately monitored. It is usual to report the presence of micro-organisms in terms of counts per ml of sample water.

In the discussion of biocides (Bott 1992), mention was made of the interference to the effectiveness of biocides by the presence of other substances and chemical compounds in the water and also the prevailing pH. Changes in microbial population may therefore be due to causes other than to toxic effects of the particular biocide in use. Along with an analysis of the microbial content of the cooling water chemical analysis should be available together with plant operating conditions.

An example of a microbial analysis is given in Table 4 showing the extent of the detail that may be necessary. Such an analysis will be carried out on a regular basis (usually once per week).

Traditionally microbial populations are monitored using either laboratory plate counts or agar dip slides. The latter have the advantage that the sample is the actual process water, but their major disadvantage is the they are not considered to be particularly accurate. On the other hand laboratory plate counts have a high degree of accuracy and repeatability, but because of the time lag between sampling and testing the results may not be representative.

The major disadvantage of these laboratory methods, already eluded to earlier, is the necessary time required to carry out the procedure which inevitably means a delay before data become available. The delay will be at least two days and usually longer. Keeping in mind the rate at which microbial populations can change, the applicability of the results becomes less useful since the data are essentially historical and therefore of limited practical application.

A leak of process fluid into the cooling water may give rise to rapid changes in microbial population, that may not be immediately apparent due to the lapsed time between sampling and availability of data. For instance the effect of a sudden leak of ammonia (a nutrient) from an ammonia cooler into the cooling water system, could cause a rapid increase in aerobic slime forming bacteria. The resulting biofilm could encourage the growth of anaerobic corrosive bacteria underneath the slime layer.

Test data related to water samples are concerned with planktonic populations, that may not be a true reflection of the extent of biofilms on surfaces within the plant. Laboratory work carried out by Bott and Miller (1983) demonstrated that provided nutrients were available, once a surface had become contaminated with bacteria in a flowing water system, rapid biofilm growth was possible even through no planktonic cells were present in the flowing water.

Clearly it would be an advantage to develop a technique that made a direct assessment of biofilm formation. Challinor (1992) lists the requirements for such a method. In summary the ideal monitor should be:

1. Rapid, ie. results within minutes rather than days.

TABLE 4. An example of the microbial analysis of a cooling water

Physical appearance: yellow liquid

	Cells/ml sample
Aerobic bacteria	
Aerobacter (Klebsiella)	20,000
Pigmented	40,000
Mucoids	< 1,000
Pseudomonas	80,000
Others	390,000
TOTAL	530,000
Anaerobic bacteria	
Sulphate reducers	< 10
TOTAL	< 10
Iron depositing bacteria	
Gallionella	None
Sphaerotilus	None
TOTAL	None
Fungi	
Moulds	< 10
Yeast	< 10
TOTAL	< 10
Algae	
Filamentous	None
Others	None
TOTAL	None
Other organisms	None

2. Capable of measuring both planktonic and sessile populations and to be able to discriminate between them.
3. Simple, requiring minimum facilities and specialist expertise.
4. Accurate.
5. Reliable.
6. Reproducible.
7. Cost effective, ie. low consumables cost.
8. Robust enough to withstand industrial conditions.

Although these comments are appropriate in many respects to any assessment of biofouling (biofilms) they are very appropriate to an instrument or piece of apparatus that may be used to obtain quantitative data.

It is unlikely that any one monitoring system will satisfy all the criteria, the final choice of method or combination of methods will therefore be a compromise.

A method based on the assay of adenosine triphosphate (ATP) by the measurement of bioluminescence produced by the luciferin-luciferase system was described by Challinor (1991) in his article. ATP which in biological systems is used to store and transmit chemical energy and is present in all viable cells, including the bacteria likely to be encountered in industrial cooling water. The technique has been widely used for carrying out field assessments of the microbial levels in water systems both in the UK and North America Young-Bandala (1983), but it needs careful and skilfull interpretation. The test can rapidly establish the presence of fouling from bacterial slimes, ie. biofilm populations together with the level of planktonic cells. The timescale of the technique is such that early corrective action may be taken in the system as necessary.

4.3 PLANT EVALUATION

In the light of the limitations associated with circulating water analysis an obvious alternative method of assessing the extent (and control) of biofilms is from plant data. Since the cooling water system involves transfer of heat, the effectiveness of the heat exchangers indicate the extent of any biofouling problem. Trends towards reduced heat transfer efficiency may be an early indication that biofilms are forming on heat exchanger surfaces. Reduced heat transfer will result in higher outlet temperatures on the process side of the heat exchanger (ie. reduced cooling due to the thermal resistance of the deposit layer). A more sensitive monitor may be the "back pressure" generated by the heat exchanger (the presence of deposits including biofilm restricts flow and adds to the frictional losses in the equipment). In power station condensers the pressure on the steam side of the condenser will give a good indication of whether or not biofouling is occurring on the cooling water side. Relatively high pressure may not be entirely due to biofilm formation, but other fouling effects. ie. the ingress of air could also give the same effect.

Sidestream monitoring may be employed. In this technique a bleed from the cooling water system as close to the inlet to the heat exchangers as possible is passed through a device containing a removable or test surface that simulates a heat exchanger surface, and on which the biofilm develops. Removal of the test coupon allows an analysis of the biofilm to be made. The extent of the biofilm is an indication of the likely development of a biofilm in the plant equipment. The side stream should operate under conditions encountered in the plant, particularly in respect of temperature and velocity. The use of a sidestream to evaluate biofilm formation in a cooling water system using removable plates has been described by Bott et al (1983). In these tests bacterial counts were made and the biofilm thickness on the plate

measured by an electrical conductivity technique. Change in weight of a test section could also be used to assess the development (or removal of biofilms).

The electrical conductance and the weighing technique both suffer from the fact that the test sections have to be removed for measurement. Ideally a monitor should be capable of continuous assessment so that trends (say in response to biocide treatment) can be established. A technique based on infra red absorbance has been used (Santos et al 1991) and the method shows promise. Continuous recording of the changes in absorbance that indicates the extent of the biofilm (thickness and compactness) is easily accomplished.

Other techniques infer the extent of biofilms by measuring changes in either pressure drop across a test section or heat transfer from a heated surface (heat flux meter). Both these measurements are amenable to continuous recording (Characklis et al 1986).

A more sophisticated investigation would be to use a model heat exchanger and record its changed heat transfer efficiency, outlet temperatures or pressure drop variations.

Many of the laboratory methods described by Bryers (1992) are applicable to side stream monitoring.

One of the uncertainties associated with side stream testing is the problem of biofilm sloughing. In response to the fluid shear on the biofilm (and the effects of biocide treatment) pieces of the biofilm can become detached from the substrate. Unless this possibility is recognised misleading information may result, particularly if "point" measurements are made.

In any plant evaluation it is necessary to have sufficient data relating to the design and operation of the equipment including: the number and type of heat exchangers, materials of construction of the exchangers and the pipework, the type of cooling tower (if installed) and its operating conditions, maximum temperatures of the cooling water, system characteristics such as water velocities, make up, and blowdown discharge.

Knowledge on how the various heat exchangers are controlled is also important in the assessment of biofilm development in order to put the monitoring procedure into proper perspective in relation to plant operation. For instance during cold weather, it is common practice in many plants to throttle back the cooling water flow entering heat exchangers to prevent over-cooling of the process stream. However the reduced flow means that the velocity through the heat exchanger is reduced that in turn promotes deposition and growth of micro-organisms on the surface. Heat transfer recovery is not always possible when the velocity is restored. Changed operating conditions in the plant should be reflected by the monitor in the sidestream.

5. Conclusion

Monitoring is essential if the cooling water is to be maintained in reasonable condition to reduce the impact of biofilm formation on plant operation. It is also required in order the minimise the cost of treatment, particularly to reduce overdosing of additives. All

feasibly possible methods of assessment and monitoring should be employed to obtain maximum information. At the same time the cost of the monitoring should be kept as low as possible.

Monitoring involves a wide ranging knowledge of all aspects of the system under scrutiny.

6. References

Bott, T.R. and Miller, P.C. (1983). *Mechanisms of biofilm formation on aluminium tubes.* J. Chem. Tech. Biotechnol. 33B, 177.

Bott, T.R., Miller, P.C. and Patel, T.D. (1983). *Biofouling in an industrial cooling water system.* Process. Biochem. 10, Jan/Feb.

Bott, T.R. (1992). *The use of biocides in industry.* In Melo, L. *et al* eds. Biofilms - Science and Technology, NATO ASI Series, Kluwer Academic Publishers, Dordrecht.

Bryers, J.D. and Characklis, W.G. (1992). *Biofilm laboratory methods: the use of flow reactors.* In Melo, L. *et al* eds. Biofilims - Science and Technology, NATO ASI Series, Kluwer Academic Publishers, Dordrecht.

Challinor, C.J. (1991). *The monitoring and control of biofouling in industrial cooling water systems.* Biofouling, 4, 4, 253.

Characklis, W.G., Zelver, N., Roe, F.I. (1986). *Continuous on-line monitoring of microbial deposition on surfaces.* Biodeterioration VI, 427.

Flemming, H.C. (1991). *Biofouling in water treatment* in Fleming, H.C. and Geesey, G.G. eds. "Biofouling and Biocorrosion in Industrial Water Systems". 47, Springer-Verlag, Berlin.

Santos, R., Callow, M.E. and Bott, T.R. (1991). *The structure of Pseudomonas fluorescens biofilms in contact with flowing systems.* Biofouling 4, 4, 319.

Van den Heuvel, J.C., De Beer, D. and Cronenberg, C.C.H. (1992). *Microelectrodes: a versatile tool in biofilm research.* In Melo, L. *et al* eds. Biofilms - Science and Technology, NATO ASI Series, Kluwer Academic Publishers, Dordrecht.

Young-Bandala, L. and Kajdasz, R. (1983). Paper 45, Int. Water Conf. Pittsburgh.

Chapter 11

REPORTS OF WORKSHOP DISCUSSIONS

METABOLISM AND ADHESION : REPORT OF WORKSHOP DISCUSSION

MADILYN FLETCHER
Center of Marine Biotechnology
Maryland Biotechnology Institute
University of Maryland System
600 E. Lombard St.
Baltimore, Maryland 21202
USA

Numerous investigations have demonstrated that the activity of microorganisms on surfaces often differs from that of freely suspended cells. However, the physicochemical, environmental, or physiological bases for such observations have rarely been demonstrated. The purpose of this workshop discussion was to explore possible explanations for some of these observations and speculate about mechanisms for altered activity.

Differences between activities of attached and free bacteria apparently are sometimes due to differences in mass transfer of nutrients to the attachment surface and to a freely suspended cell. Flow over a surface results in a greater mass transfer of nutrients to microorganisms on the surface. However, this process probably cannot account for all the differences that have been observed between microorganisms residing in biofilms and those that remain unattached.

It is possible that attached bacteria may "sense" conditions at the surface, and may respond through alterations in physiology. For example, hydrodynamic forces, such as shear stress, could influence microbial processes within a biofilm, including the synthesis of extracellular polymers. Possibly, microorganisms have evolved the ability to synthesize adhesive polymers in response to surface contact or to modify synthesis in response to hydrodynamic conditions. Biofilm thickness, density, and stability have all been found to vary when the biofilms developed under different flow rates. Under high flow rates (high shear stress) biofilms have been found to be more condensed, have lower mass transfer coefficients, and be more stable than biofilms that developed under lower flow rates. These observations suggest that the chemical composition or configuration of the biofilm polymer is different according to flow conditions.

Is it possible that microorganisms are able to alter polymer synthesis in response to hydrodynamic conditions, or is the observed difference in polymer properties due to variations in compression the biofilm is subjected to during synthesis? There is evidence that the extracellular polysaccharides synthesized by diatoms tend to be sulfated under turbulent conditions and less sulfated and more carboxylated as turbulence decreases. Such chemical changes have functional implications, as sulfated polysaccharides tend to

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 673–675.

be more flexible and more resilient to stress. Analogous modifications in polymer structure might also occur with bacterial polymers. Such variations in resilience could be measured as changes in viscoelasticity, and, thus, it should be possible to experimentally quantify changes in polymer properties that occur in response to altered environmental conditions, e.g., shear stress, nutrient conditions. There is a fundamental need for experimental characterization of the chemical and structural properties of biofilm polymers and how they are modified in response to specific hydrodynamic conditions.

Alterations in nutrient conditions frequently result in variations in extracellular polymer characteristics. Therefore, if changes in hydrodynamic conditions are accompanied by changes in nutrient availability or composition, bacterial biofilm characteristics could be affected. Different types of polymer have been shown to be synthesized during log or stationary phases in batch culture. Biofilm polymers have been shown to lose structural strength when nutrients become depleted. Changes in both cell wall composition and organism morphology have been observed with yeast under different growth conditions. Clearly, the effects of nutrients on biofilm development and chemistry are complex, and the physiological state of microorganisms must be carefully considered when carrying out experiments on biofilm structure and stability.

Although there is evidence that nutritional and hydrodynamic factors can influence responses of biofilm bacteria, there is only one documented example of a microorganism being able to "sense" the attachment substratum. Some vibrios are induced to produce laterial flagella when motion of the polar flagellum is impeded by cell attachment to the surface. Thus, in this case, the bacterium apparently senses the surface by interference with flagellum rotation. Are there other mechanisms by which microorganisms could sense a surface? One possibility is deformation of the cell envelope that results from attractive forces between the cell and attachment surfaces. Assuming that this deformation was translated to the cytoplasmic membrane, this could provide a mechanistic basis for bacterial responses to a solid surface. The cytoplasmic membrane is semi-permeable to ions, and thus will act as a small capacitor. A deformation would then induce a local change in the capacity, and this would cause a transient electric current to equalize the membrane potential. This, in turn, would effect a permanent change in ionic concentrations locally within the membrane. Changes in ion concentrations, e.g., K, Ca, have known signal functions in many organisms, and it is worth speculating about their potential function in microbial recognition of solid surfaces.

In the experimental investigation of the effects of solid surfaces on microbial activity, there are a number of important points to bear in mind. First, there are numerous measures of bacterial activity, ranging from extracellular polymer synthesis, to cell growth, to substrate uptake, to concentration of biomass components, such as ATP. To really understand the impact of attachment on biofilm physiology, it is important to measure the aspect of activity that relates to the hypothesis being addressed. Bacterial growth is rarely, if ever, balanced in nature, and different measurements of activity cannot be expected to correlate precisely with one another.

The second point to consider when investigating the effects of attachment on bacterial metabolism is not to always assume that life on the surface is the best option. In some environments, conditions in the bulk phase may be just as advantageous, if not moreso, than those on the surface. Otherwise, bacteria would have exclusively adapted to a biofilm mode of existence. Thus, bacterial activity in biofilms or on surfaces is not necessarily greater than that of the freely swimming bacteria. But, in many, if not most, cases, biofilm activity *is different*. The mechanistic bases and ecological implications of these various modifications in metabolism are poorly understood, and a multidisciplinary effort is required for their elucidation.

ACKNOWLEDGMENTS

This workshop summary includes the ideas, observations, and speculations of many participants. Key contributors included Luis Melo, Nava Mozes, Jean Luc Rols, Hans-Curt Flemming, J.C. van den Heuvel, Manuel Mota, Rosario Oliveira, T.R. Bott, A.H. Chamberlain, James Bryers, and Keith Cooksey, as well as others who made invaluable contributions to this discussion.

PRACTICAL NEEDS FOR BASIC RESEARCH: A BASIS FOR INTERACTION BETWEEN MICROBIOLOGISTS AND ENGINEERS

K.E. COOKSEY
Montana State University
Bozeman, MT 59717

1. Preamble

The NATO Advanced Study Institute on "Biofilms: Science and Technology" attracted mixed participation from practitioners of both engineering and microbiology. Thus it provided an opportunity to explore the universally perceived reasons that the two groups interacted poorly. What follows is a distillation of the ideas expressed from the floor and chair during a discussion workshop.

2. Introduction

The rationale for scientific endeavor is the pursuit of knowledge, whereas the raison d'être for engineering is to solve human problems. In idealistic terms, science is the pursuit of knowledge for its own sake, with no profit motive. Engineering, on the other hand is driven entirely by human needs. An engineer builds a bridge to carry people. There are no purely scientific reasons to build such a structure. These differences in philosophy between scientists and engineers are reflected in the education systems that train them and leads to a certain amount of professional intransigence. Scientists often believe that engineers hold a narrow view of the world. On the other side of the coin, engineers frequently think scientists are unfocused. There is some truth to both of these views.

None of this would matter if the two professions never needed to interact. In the past, many engineering problems were solved by following an empirical approach, i.e. careful observation of conditions that promoted an engineering failure lead logically to the means to prevent it. This is not scientific research. Nowadays many of the problems facing not only the engineering community, but all of us require the

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 677–681.

application of new knowledge for their solution. It is these particular areas where novel information is required that are potentially the most fruitful for interdisciplinary collaboration.

Funding agencies in the European Community commonly promote scientific interaction with the business sector and the involvement of universities in solving industrial problems. This philosophy for the funding of scientific research is carried out to a far lesser extent in the United States, but programs where industry and the academic sector are encouraged to interact are increasing. If nothing else, collaboration then becomes one means of professional survival!

3. Discussion

The discussion followed a format wherein the chair suggested a topic and then asked for comment from the floor.

3.1. DETACHMENT OF BIOFILMS

The process of detachment of biofilms is poorly understood and this complicates the formulation of satisfactory models for net biofilm processes. There is a poor correlation, for instance, between detachment and shear force. The correlation is also unsatisfactory between shear and biofilm thickness. Much of what we know is dependent on recovery of detached cells as viable counts rather than cellular carbon. A further complicating factor is that the growth rate of the biofilm influences the ease with which a biofilm detaches. In large scale hollow-fibre reactors used to grow mammalian cells detachment occurs when all the space able to be occupied by cells is filled, i.e. yet another factor promoting detachment. Detachment of areas of a uniform biofilm leads to spatial discontinuities in redox potential and the creation of electrochemical concentration cells. Such cells can be involved in electrochemical corrosion.

The problems in understanding detachment appear to be exacerbated by the number of variables in the development of a biofilm at a surface and the influence of those variables on biofilm cohesive strength. Thus, in detachment studies, factors such as nutrient flux should be controlled independently of shear force even though both are dependent on flow rates over the surface. There were no suggestions concerning how this might be achieved. Furthermore, it is important to study the quality as well as the quantity of detached biofilm. For instance, rarely is the size of the detached particle measured. Whether the particle is a single cell or an aggregate of many cells is important in stoichiometric and physiological descriptions of biofilm

development. Detachment of biofilms is of highly practical importance in the loss of catalysts in bioreactors, the contamination of parenteral water and the spread of *Legionella* infection from air-conditioning ducts.

The physical means by which films detach appears controversial. Previously, it was considered that turbulent bursts that transcend the viscous sub-layer were responsible to generate the forces necessary to remove a biofilm from a surface. Now it is thought that such bursts do not have sufficient power to achieve this. The film is viscoelastic not rigid, and this contributes to its resistance to turbulent bursts. It seems that this is an area where new research is needed, however, the experimental data (high speed films) would not be simple to acquire.

3.2 MODELLING AND FLOW CONSIDERATIONS

Industrial interest in modelling is almost completely in its ability to predict biofilm effects. Several discussants mentioned the need for rapid testing in industry. In the laboratory, flow testing regimes are not rapid and are expensive to replicate. Thus, there is need to design equipment that monitors biofouling in situ. Such systems will most likely be installed in side- stream pipes. The potential inadequacy of this approach is that the flow rates in the main and side-streams have to be identical and this is difficult to achieve in an industrial situation.

It was stressed that flow is a crucial parameter in biofilm accumulation and usually laboratory studies did not mimic the real world situation. Whereas the laboratory investigator controls flow rather precisely, in nature flow rates over a surface vary enormously with time -- and since flow controls nutrient flux, this varies too. Modelling exercises will be complicated by such variations.

3.3 SPECIFIC PROFESSIONAL NEEDS FOR INTERACTION

The above discussion was contributed about equally by microbiologists and engineers. The chair asked how microbiologists could increase the relevance of their research to the engineering community. The following points were raised. Variations in reporting cell numbers (cell number itself, DNA content, other biomass estimates such as ATP) caused engineers difficulty when trying to compare results. Another problem was that microbiologists often report the existence of phenomena, but neglect to measure the rates at which they occur. Thus, their significance cannot be assessed easily. Engineers (especially chemical engineers) use material balance equations to track the progress of a process and recommend the approach to microbiologists.

3.4 LESS WELL KNOWN CASES OF PROBLEMS CAUSED BY BIOFILMS

Several of the engineering papers at the A.S.I. concerned increases in heat transfer resistance caused by biofouling. This is a well known problem in this industry. The chair asked for the names of other industries where the importance of problems caused by biofilms was still emerging. Suggestions were: the pulp and paper industry, parenteral drug industry, in-stream sensors (many industries), food (especially in Europe where food storage patterns are changing), space vehicles (potable water, sewage), and the toxic waste remediation industry (fouled wells, bioconcentration of toxic materials in biofilms in extraction wells).

3.5 VIRAL PROBLEMS IN BIOFILMS : DO THEY EXIST ?

All high density cultures in nature attract parasites, whether this be monoculture in a field or in a fixed film reactor. The presence of viral particles in films is not well documented. Few people have seen such particles, but then viruses can be seen only by electron microscopy and this tool is not used as a routine monitor in industry. Unexplained massive sloughing events have been noticed however, and these may be attributable to viral infection.

3.6 GROWTH MODELS

All of the growth models presented at the A.S.I. used the equations developed by Monod. It appears they are not sensitive to changing parameters in the chemostat. This is an example where the microbiologist can help the modeler directly in providing more accurate descriptions of microbial growth. It was pointed out that although Monod's equations are the ones favored by engineers, but there are several other models in the literature. For instance, the model according to Droop considers the cell membrane as a site of mass transfer resistance whose contribution to the overall mass transfer can not be described in terms of diffusion. Transmembrane transport is often an active, not a passive phenomenum. Growth then is better described in terms of the fraction of the external substrate inside the cell, not its exterior concentration.

3.7 GRADUATE STUDENT PERCEPTIONS

Discussion in the final period of the meeting came almost entirely from graduate students. They were asked collectively what they considered to be the major impediments to their involvement in interdisciplinary research. All blamed the

defined niches they are expected to inhabit during their highly structured education. It was pointed out that an appreciation for the importance of interdisciplinary associations is more easily obtained in a Polytechnic institute rather than a university. Many favored interdisciplinary degrees, but were wary of the epithet "Jack of all trades, master of none". At fault too are the professional engineering organizations that restrict full membership to those who have followed traditional (i.e. structured) patterns of education. Membership in professional associations is sometimes a condition and employment in industry. Professional scientific societies are usually not so restrictive and require only an interest in the subject as a membership condition.

The discussion was summarized succinctly by a young chemical engineer who had taken several courses in microbiology, including molecular biology. When talking to engineers she told us that she knew more than they did, but when talking to molecular biologists, she realized that they knew more than she did. Perhaps the problems of interaction are only those of the "old guard", the younger generation doesn't seem to possess the same inhibitions!

BIOFILMS IN INDUSTRIAL WATER AND WASTEWATER SYSTEMS SUMMARY OF DISCUSSION

HAN VAN DEN HEUVEL
University of Amsterdam
Chemical Engineering Department
The Netherlands

KALMINDER KAUR
University of Birmingham
Chemical Engineering School
United Kingdom

Subject of discussion : "Studies on less defined wastewater treatment systems" versus "More fundamental research on biofilm processes".
Conclusions : Industries are conservative with respect to water and wastewater treatment. Legislation is the main cause for new developments.

Subject of discussion : "Heat exchanger design on a thermal basis and the use of safety factors for biofouling" versus "Direct account of biofouling on a mechanistic basis in the design phase".
Conclusions : Anti-fouling equipment and procedures are of equal (economic) importance and should therefore have equal emphasis from the beginning. Both biological and engineering subjects should be incorporated in educational programmes.

Subject of discussion : "Importance of mass transfer at gas-liquid interfaces in complex media (such as animal cell cultures)" versus "Emphasis on liquid-biofilm-support interfaces".
Conclusions : No significant response, i.e., the audience did not seem to support the importance of gas-liquid interfaces in general,except for their possible influence on the detachment of parts of the biofilm.

Subject of discussion : "Shock-loading and operation of wastewater treatment plants" versus "Design and the use of safety factors".
Conclusions : Fundamental knowledge of biofilm processes at the level of physiology and mass transfer is required for optimal design. Good housekeeping (involvement of all levels of personel) to prevent the production of (highly loaded) wastewater is of equal importance.

Subject of discussion : "Fundamental studies on physico-chemical behaviour of cell walls is highly important" versus "Characteristics of a single species are not significant in a complex multi-species environment".
Conclusions : Participants acknowledge the relevance of physico-chemical changes in well defined systems, but not for industrial water and wastewater treatment systems.

L.F. Melo et al. (eds.), Biofilms – Science and Technology, 683–684.

General Conclusions not covered in the foregoing summary :

- For both heat exchangers and wastewater treatment, operator information should be communicated towards designers.
- Pilot-plant studies on new concepts in water and wastewater treatment should be encouraged fron a pragmatic point of view.

Topics for discussion proposed by : Carel Cronenberg, Jim Chenoweth, Anita Handa-Corrigan, Antonio Brito, R. Gage.

GLOSSARY

GLOSSARY

Antagonistic interactions - interactions between populations of two species of microorganisms in which one or both populations suffers, e.g. predation, parasitism.

ATP - adenosine triphosphate; a principal carrier of energy and phosphate in biological metabolism.

Autotrophs - organisms that do not require external sources of organic compounds; carbon is obtained from CO_2 and energy is obtained from light or from the oxidation of inorganic compounds.

Blowdown - the removal of water from a system where evaporation takes place (cooling water tower; spray pond) to maintain the concentration of the dissolved solids within specified limits to reduce scale formation.

CFU - colony froming units.Used to express the number of bacteria present by determining the number of colonies formed by plating dispersed bacterial suspensions on solid media.

Chemiosmotic theory - basis of energy generation by bacteria and cells of higher organisms that depends upon a proton gradient and maintenance of membrane potential across the cytoplasmic membrane.

Competitive interactions -interactions between populations of two species of microorganisms that require the same resource,e.g., space, a limiting nutrient, resulting in suboptimal growth of both populations.

Consortium - two or more bacterial species with complementary physiologies that live in close association and interact in a positive manner.

Cyanobacteria - a type of photosynthetic bacterium containing chlorophyll *a*; previously known as blue-green algae.

Dental plaque - the complex microbial community found on tooth surfaces.

Gingiva - the gum; the fibrous tissue covered by mucous membrane which covers the alveolar processes of the upper and lower jaws and surrounds the necks of the teeth.

Glycocalyx - polysaccharide-containing structures produced by bacteria and lying outside the outer membrane of Gram-negative bacteria or the peptidoglycan of Gram-positive.

Heterotrophs - organisms requiring organic compounds as sources of carbon and energy.

Homeostasis - maintenance of stability through a balance of interactions that offset imposed fluctuations; can refer to environmental interactions and stability or to physiological stability within an organism.

Hybridization - a means of detecting and identifying specific nucleic acid sequences of DNA or RNA by their annealing (hybridizing) with complementary, known nucleic acid sequences that have been tagged with a radioisotope or fluorochrome.

Hydroxypatite - the main mineral component of teeth, represented by the chemical formula $Ca_{10}(PO_4)_6(OH)_2$.

Immunofluoerescent labelling - ue of antigens or antibodies, which have been tagged with a fluorescent dye, to detect homologous antibodies or antigens.

In vitro - in glass;a reaction or process that occurs in a vessel in the laboratory.

In vivo - in the living organism.

IPTG - isopropylthio-beta-D-galactoside; in molecular biology, it is often used to induce production of beta-galactisodase.

Make up water - in cooling water systems water is lost from the system by a number of methods but principally evaporation (cooling towers or spray ponds). In order to maintain water volume the water loss is replaced by "make up" water from the raw water source (river, lake or bore hole).

Mucosa (alveolar mucosa) - lining of the oral cavity

Mutualistic interactions - interactions between populations of two microorganisms by which both populations benefit.

Periodontal disease - general term for several diseases in which the supporting tissues of teeth are attacked.

Photorespiration - light-induced increase in algal respiration.

Plasmid - DNA which is not part of the chromosome, resides in the cytoplasm, and is capable of independent replication; often carries genes that are not essential to the physiology of the cell, e.g. genes conferring antibiotic resistance.

Plasmid copy number - number of copies of a given plasmid per cell.

Recombinant - a nucleic acid sequence that contains a new combination of DNA sequences; often applied to engineered DNA, in which sequences are combined to obtain a desired product, e.g. recombinant DNA.

Reynolds number - the dimensionless number is a measure of the ratio of momentum to viscous forces within a flowing system. For a fluid flowing in a tube, the Reynolds number is given by the product of fluid velocity, tube diameter and fluid density, divided by fluid viscosity. In pipe flow it is generally accepted that laminar flow exists below a Reynolds number of 2000, whereas above this value turbulent eddies start to appear.

RNA polymerase - the enzyme which transcribes DNA into RNA, in the process by which RNA that is complementary to the DNA is synthesized.

Slats - in order to facilitate evaporation in a cooling tower, extensive surface (over which the water flows) is presented to the air stream. The simplest arrangement is to have supported planks of wood known as slats, across the width of the tower.

Synergistic interactions - an interactive association between two populations of micro-organisms which is not necessary, but by which each population benefits.

Syntrophic - applied to two or more populations of microorganisms that supply each other's nutritional requirements.

Transconjugants - bacteria that have received DNA by the process of conjugation, a process by which a plasmid is transferred from one cell to another.

Transcription - the process of synthesis of RNA that is complementary to DNA; the first step in expression of a gene.

Transposon mutagenesis - technique of generating mutants through the use of a transposon, a piece of DNA that can insert itself into a gene, thereby interupting the gene sequence and preventing its expression.

Vector - generally a plasmid or bacteriophage, into which fragments of DNA can be inserted and which can be used to transport that DNA into a recipient bacterium.

SUBJECT INDEX

C

E

H

Q

R

S